# Crosstalk between the androgen receptor and progesterone receptor isoforms in breast and prostate cancer

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# Declaration

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# Abstract

Breast and prostate cancer growth and survival are dependent on signalling via the estrogen receptor (ER) and androgen receptor (AR), respectively. However, other steroid receptors such as the progesterone receptor (PR), are also implicated in both cancers, and emerging evidence suggests considerable crosstalk between these steroid receptors in breast cancer. Investigations into similar crosstalk mechanisms are lacking in prostate cancer. As the AR and PR are likely co-expressed in a subset of breast and prostate cancers, it is surprising that no studies have investigated crosstalk between the AR and PR in these cancers. Both these receptors can activate transcription by binding to DNA on a classical response element, termed either the progesterone response element (PRE) when the PR is bound, or the classical androgen response element (ARE) when the AR is bound. However, the AR can also bind to an AR-selective ARE as well as to the ER binding site, termed the estrogen response element (ERE). Whether the PR isoforms, PRA and PRB, can similarly activate the AR-selective ARE and ERE is not known. In this study, we investigated whether the PR isoforms, in the absence and presence of known PR agonists (synthetic promegestone (R5020), natural progesterone ($P_4$), and synthetic progestin medroxyprogesterone acetate (MPA)), could modulate the transactivation function of the AR via the above-mentioned response elements in the MDA-MB-231 breast cancer and PC3 prostate cancer cell lines. The cells were transiently transfected with the expression vectors for the AR and/or PR isoforms, together with the applicable promoter-reporter constructs. The general trend observed was that both the unliganded and liganded PR isoforms augmented AR activity in a cell line-, ligand- and/or promoter-specific manner. Specifically, we showed that PRB, both in the absence and presence of PR ligands, generally upregulated AR transactivation on the various response elements in the breast and prostate cancer cells. While AR transactivation function was also increased by PRA on the selective ARE and ERE, PRA decreased AR-mediated transactivation on the classical ARE. We also provide novel evidence that both PR isoforms mimic AR activity on the selective ARE and the ERE in both cell lines, which may provide a mechanism through which the PR mediates oncogenic effects in both cancers. We did not observe cell proliferation in the presence of 5α-dihydrotestosterone (DHT) in either cell line transfected with the AR under the experimental conditions used in this study. In summary, even though the results from this study are preliminary, we are the first to show that the transactivation function of the AR is generally enhanced in the presence of the PR isoforms in both breast and prostate cancer. These findings support a potential crosstalk mechanism between the AR and PR isoforms in these cancers. Although the precise physiological implications of these results require further investigation, our findings contribute to the understanding of crosstalk between steroid receptors, particularly the AR and the PR isoforms, and how this may influence breast and prostate cancer cell growth.

# Opsomming

Die groei en oorlewing van bors- en prostaatkanker is afhanklik van seine deur onderskeidelik die estrogeenreseptor (ER) en androgeenreseptor (AR). Ander steroïedreseptore soos die progesteroonreseptor (PR) is egter ook betrokke in beide kankers, en onlangse bewyse stel voor dat aansienlike wisselwerking tussen hierdie reseptore in borskanker voorkom. Ondersoeke na soortgelyke wisselwerkingsmeganismes in prostaatkanker ontbreek. Aangesien die AR en PR waarskynlik saam uitgedruk word in ‘n onderafdeling van bors- en prostaatkankers, is dit verbasend dat geen studies die wisselwerking tussen die AR en PR in hierdie kankers ondersoek het nie. Beide hierdie reseptore kan transkripsie aktiveer deur te bind aan DNS op ‘n klassieke responselement, benoem òf die progesteroonresponselement (PRE) wanneer die PR bind, òf die klassieke androgeenresponselement (ARE) wanneer die AR bind. Die AR kan egter ook bind aan ‘n AR selektiewe ARE asook die ER bindingsarea, benoem die estrogeenresponselement (ERE). Dit is onbekend of die PR isoforme, PRA en PRB, die AR selektiewe ARE en ERE soorgelyk kan aktiveer. In hierdie studie, het ons ondersoek of the PR isoforme, in die afwesigheid en teenwoordigheid van bekende PR agoniste (sintetiese promegestoon (R5020), natuurlike progesteroon (P4), en die sintetiese progestien medroksieprogesteroonasetaat (MPA)), die transaktiveringsfunksie van die AR deur die bogenoemde responselemente kon wysig in die MDA-MB-231 borskanker- en PC3 prostaatkankersellyne. Die selle was tydelik getransfekteer met die uitdrukkingsvektor vir die AR en/of PR isoforme, saam met die toepaslike promotor-rapporteerder konstrukte. Die algemene tendens wat waargeneem is, was dat beide die ligandlose en ligand-gebonde PR isoforme die aktiwiteit van die AR verhoog het in ‘n sellyn-, ligand- en/of promotor-spesifieke manier. Ons het spesifiek getoon dat PRB, beide in die afwesigheid en teenwoordigheid van PR ligande, in die algemeen AR transaktivering op verskeie responselemente in die bors- en prostaatkankerselle opreguleer. Alhoewel die AR transaktiveringsfunksie ook deur PRA verhoog was op die selektiewe ARE en ERE, het PRA die AR-bemiddelde transaktivering op die klassieke ARE verlaag. Ons het ook nuwe bewyse voorsien dat beide PR isoforme die aktiwiteit van die AR naboots op die seletiewe ARE en ERE in beide sellyne, wat ‘n meganisme mag voorsien waardeur die PR onkogeniese effekte in beide kankers kan uitvoer. Onder hierdie gebruikte eksperimentele kondisies, het ons geen selproliferasie in die teenwoordigheid van 5α-dihidrotestosteroon (DHT) in enige sellyn getransfekteer met die AR waargeneem nie. In opsomming, alhoewel die resultate van hierdie studie voorlopig is, is ons die eerste om te toon dat die transaktiveringfunksie van die AR oor die algemeen verhoog is in die teenwoordigheid van die PR isoforme in beide bors- en prostaatkanker. Hierdie bevindinge ondersteun die potensiële wisselwerkingsmeganisme tussen die AR en PR isoforme in hierdie kankers. Alhoewel die presiese fisiologiese implikasies van hierdie resultate verdere

ondersoek verlang, dra ons bevindinge by tot die begrip van wisselwerking tussen steroïdreseptore, veral die AR en die PR isoforme, en hoe dit bors- en prostaankankergroei mag beïnvloed.

***Solvitur ambulando*** - It is solved by walking

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# Alphabetical list of abbreviations

| | |
|---|---|
| αERKO | estrogen receptor alpha knockout |
| βERKO | estrogen receptor beta knockout |
| AF | activation function |
| ANOVA | analysis of variance |
| AR | androgen receptor |
| ARE(s) | androgen response element(s) |
| ARE-luc | androgen response element-luciferase |
| ARKO | androgen receptor knockout |
| ATCC | American Type Culture Collection |
| CAF | Central Analytical Facility |
| cDNA | complementary deoxyribonucleic acid |
| CFP | cyano fluorescent protein |
| ChIP | chromatin immunoprecipitation |
| CRPC | castration-resistant prostate cancer |
| CS-FCS | charcoal-stripped fetal calf serum |
| CYP17A1 | cytochrome P450 17A1 |
| CYP19A1 | cytochrome P450 19A1/aromatase |
| DBD | DNA-binding domain |
| DES | diethylstilbestrol |
| Dex | dexamethasone |
| DHT | 5α-dihydrotestosterone |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulphoxide |
| DNA | deoxyribonucleic acid |
| $E_2$ | 17β-estradiol |
| ECL | enhanced chemiluminescence |
| EDTA | ethylene-diaminetetra-acetic acid |
| ERα | estrogen receptor alpha |
| ERβ | estrogen receptor beta |
| ER | estrogen receptor |

| | |
|---|---|
| ERE(s) | estrogen response element(s) |
| ERE-luc | estrogen response element-luciferase |
| ERG | E26 transformation-specific (ETS)-regulated gene |
| EtOH | ethanol |
| ETS | E26 transformation-specific |
| FCS | fetal calf serum |
| FRET | Förster resonance energy transfer |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GR | glucocorticoid receptor |
| h | hinge region |
| HPA | hypothalamus-pituitary-adrenal |
| HRP | horseradish peroxidase |
| HT | hormone therapy |
| kb | kilobase pairs |
| kDa | kiloDaltons |
| KLK | kallikrein |
| LB | Luria Bertani |
| LBD | ligand-binding domain |
| Mib | mibolerone |
| MMTV | mouse mammary tumour virus |
| MPA | medroxyprogesterone acetate |
| MR | mineralocorticoid receptor |
| mRNA | messenger ribonucleic acid |
| MTT | 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide |
| NFκB | nuclear factor kappa B |
| NTD | N-terminal transactivation domain |
| $P_4$ | progesterone |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PR | progesterone receptor |
| PRA | progesterone receptor isoform A |

| | |
|---|---|
| PRB | progesterone receptor isoform B |
| PRE(s) | progesterone response element(s) |
| PRE-luc | progesterone response element-luciferase |
| PSA | protein-specific antigen |
| qPCR | quantitative real-time polymerase chain reaction |
| R5020 | promegestone |
| re-ChIP | sequential chromatin immunoprecipitation |
| RLU | relative light units |
| RPMI | Roswell Park Memorial Institute |
| RU486 | mifepristone |
| S | steroid |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |
| SERD(s) | selective estrogen receptor downregulator(s) |
| SERM(s) | selective estrogen receptor modulator(s) |
| siRNA | small interfering RNA |
| SOC | super optimal broth medium with catabolite repression |
| SPRM(s) | selective progesterone receptor modulator(s) |
| SR | steroid receptor |
| SRE | steroid response element |
| TBS | tris buffered saline |
| TBST | tris buffered saline tween |
| TE | tris ethylene-diaminetetra-acetic acid |
| TNBC | triple-negative breast cancer |
| YFP | yellow fluorescent protein |

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# Chapter 1

# Literature review

## 1.1 Introduction

Globally, breast cancer is the most common cancer in women, and prostate cancer the second most common cancer in men (Ferlay et al., 2015; Torre et al., 2015). Both breast and prostate cancer are hormone-dependent malignancies, relying on steroid hormones such as estrogens and androgens, respectively, for survival (Sommer and Fuqua, 2001; Shafi et al., 2013). Indeed, estrogen and its cognate receptor, the estrogen receptor (ER), are considered the main etiological factors contributing to breast cancer development (Sommer and Fuqua, 2001). Prostate cancer, on the other hand, is dependent on androgen signalling via its cognate receptor, the androgen receptor (AR) (Lee et al., 2003; Azzouni and Mohler, 2012). Current therapies for breast cancer thus target estrogen biosynthesis and the ER, while prostate cancer treatments target the AR and androgen biosynthesis (Nagaraj and Ma, 2015; Attard et al., 2016). Resistance to therapy, however, is a pressing concern in both breast and prostate cancer (Rau et al., 2005). Interestingly, the ER subtype, ERα, is also implicated in prostate cancer development and progression (Bonkhoff et al., 1999). Similarly, AR expression in ER-negative breast cancer tumours is associated with breast cancer development (Peters et al., 2009). Numerous studies are thus focusing on the role of estrogens and the ER in prostate cancer and androgens and the AR in breast cancer (Nelles et al., 2011; Cochrane et al., 2014; Yeh et al., 2014; Omoto and Iwase, 2015; Wellberg et al., 2017). In addition to the ER and AR, other steroid receptors such as the progesterone receptor (PR) are also expressed in breast and prostate tumours (Bonkhoff and Berges, 2009; Knutson and Lange, 2014). Considering that the PR is expressed in both breast and prostate cancer, and that studies have shown that the PR plays an important role in both diseases (Bonkhoff et al., 2001; Mc Cormack et al., 2007; Grindstad et al., 2015), it is surprising that current treatments do not target this receptor (Rau et al., 2005). Interestingly, a number of studies have found that steroid receptor crosstalk between ERα and the AR, as well as ERα and the PR, plays an integral role in breast cancer (Kumar et al., 1994; Panet-Raymond et al., 2000; Peters et al., 2009; Muthusamy et al., 2011; Grubisha and DeFranco, 2013; Cochrane et al., 2014; D'Amato et al., 2016). Similar studies investigating steroid receptor crosstalk mechanisms in prostate cancer are scarce. Whether crosstalk between the PR and AR occurs in breast and prostate cancer, and the implications of such crosstalk, has not been investigated. Understanding crosstalk between these steroid receptor signalling pathways may therefore contribute to the development of new improved therapies for the treatment of breast and prostate cancer. The primary aim of this review is to describe the mechanism of action of the AR and PR, and their ligands, in breast and prostate cancer, highlighting similarities and differences. Considering the increasing importance of steroid receptor crosstalk in breast cancer, known crosstalk mechanisms and their implications will also be discussed.

## 1.2 General structure and mechanism of action of steroid receptors

The steroid hormone receptor family includes the glucocorticoid receptor (GR), mineralocorticoid receptor (MR), ER, PR and AR (Tata, 2002; Robinson-Rechavi, 2003). These receptors are ligand-activated transcription factors with a highly conserved structure (Tata, 2002), containing an upstream N-terminal transactivation domain (NTD) with activation function-1 (AF-1), a central DNA binding domain (DBD), a downstream hinge region (h) and a C- terminal ligand binding domain (LBD) which contains the activation function-2 (AF-2) domain (Fig. 1.1). The AF-1 domain is responsible for ligand-independent activation of the receptors, while the AF-2 domain mediates ligand-dependent effects (Wärnmark et al., 2003; Lavery and McEwan, 2005).

The ER exists as two main subtypes, ERα and ERβ, which are expressed from two different genes (Kuiper et al., 1996). Although ERα and ERβ only share a 47% overall sequence identity, the DBD domain is highly conserved and has a 94% sequence identity (Muramatsu and Inoue, 2000). Similarly, the PR exists as two isoforms, PRA and PRB, which are transcribed from two different promoters of a single gene (Kastner et al., 1990). The PR isoforms are identical in sequence except that PRB contains an additional 164 amino acids in the NTD. An additional AF domain, namely activation function-3 (AF-3), is found in this NTD, rendering PRB more transcriptionally active than PRA in the presence of ligand (Sartorius et al., 1994). Notably, this difference in activity is due to the fact that the ligand induces a conformational change in PRB such that the AF subdomains are able to functionally interact (Tung et al., 2001; Takimoto et al., 2003). Although two separate isoforms transcribed from the same gene have also been reported for the AR, these isoforms are not well described (Lavery and McEwan, 2005; Azzouni and Mohler, 2012).

When comparing the structures of the ER, PR and AR (Fig. 1.1), it is clear that the PR isoforms are more similar to the AR as they share a 82% and 55% amino acid sequence identity in the DBD and LBD, respectively (Gao et al., 2005). In comparison, the ER subtypes are the most distinct (Gao et al., 2005) with the amino acid sequence identity of ERα and ERβ to the AR only 59% and 22-25% in the DBD and LBD, respectively (Gao et al., 2005). Furthermore, although not indicated in figure 1.1, the PR shares a 54% and 23% amino acid sequence identity with the ER subtypes in the DBD and LBD, respectively (Ruff et al., 2000). As the PR and AR share a high degree of structural homology, these receptors recognize and bind similar DNA sequences, while the ER subtypes bind distinct DNA motifs (Beato, 1989).

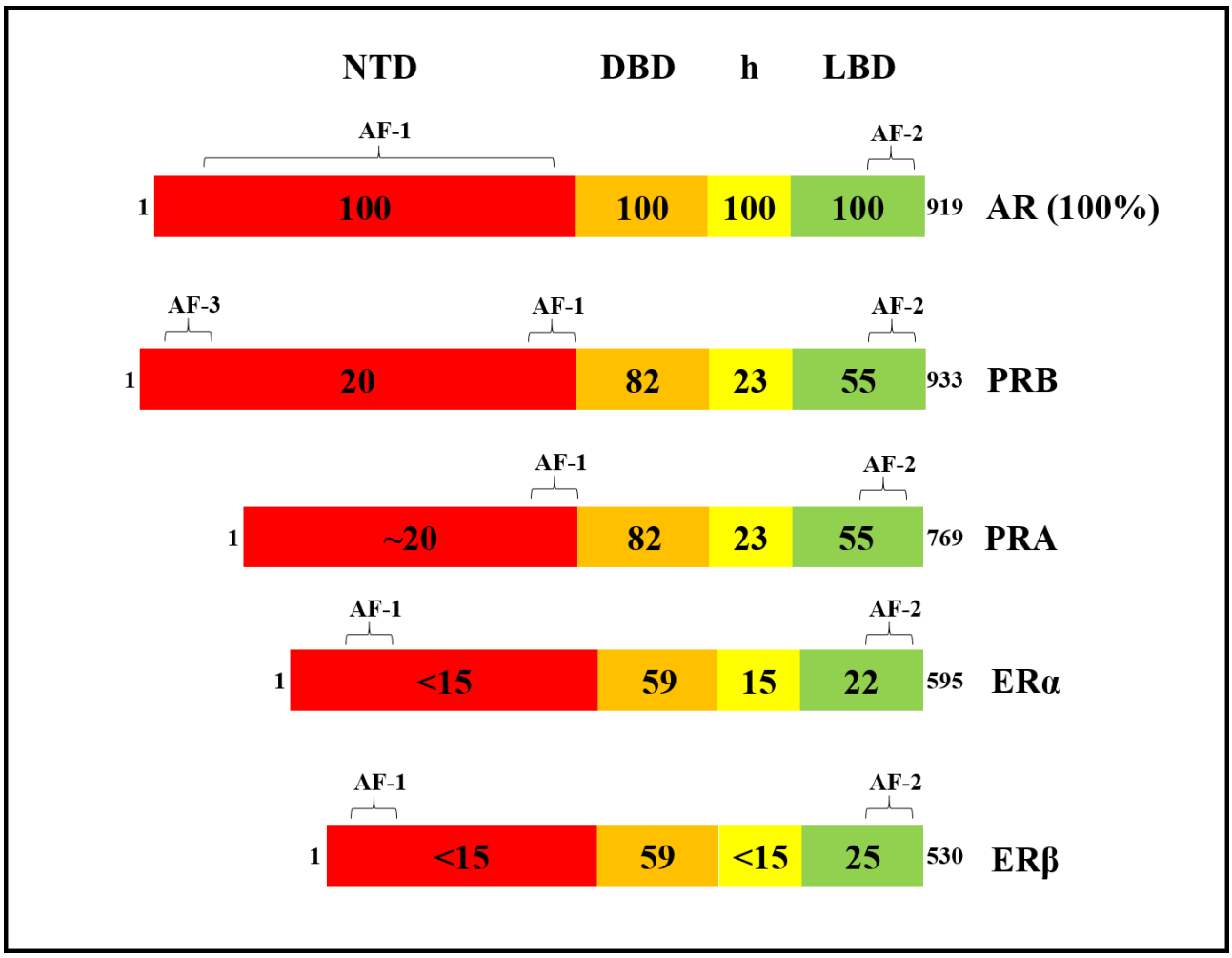


**Figure 1.1: Comparison of the structural domains of the AR, PR isoforms and ER subtypes.** The numbers indicated within the structure represent the amino acid sequence identity of each domain to the AR (set as 100%), while those outside of the structure represent the total number of amino acids. The N-terminal transactivation domain (NTD) contains the activation function-1 (AF-1) domain, while the activation function-2 (AF-2) domain is located in the LBD. The additional activation function 3 (AF-3) domain in PRB is also shown. The DNA-binding domain (DBD) confers the sequence-specificity of protein-DNA interactions, while the downstream hinge region (h) and ligand-binding domain (LBD) contribute to the ligand-specificity of the receptors (Tata, 2002). Figure adapted from: Tata, 2002; Robinson-Rechavi, 2003; Gao et al., 2005.

Steroid receptors are generally located in the nucleus, bound to chaperone proteins which stabilise and prevent degradation of the receptor (Fig. 1.2) (Pratt et al., 2004). While the unliganded AR and PRB are primarily localised in the cytoplasm, the unliganded ER subtypes and PRA are present in the nucleus (Leslie et al., 2005; Echeverria and Picard, 2010). Upon ligand binding, steroid receptors undergo a conformational change and translocate to the nucleus where they can activate (transactivation) or repress (transrepression) gene expression (Tata, 2002; Robinson-Rechavi, 2003). During transactivation, the steroid receptor binds as a dimer, directly to the DNA, at specific sequences termed steroid response elements (Tata 2002, Robinson 2003). Transrepression on the other hand is a process whereby the liganded steroid receptor tethers to other DNA-bound

transcription factors, such as nuclear factor kappa B (NFκB) (Tata, 2002; Robinson-Rechavi, 2003). The transcriptional activity of the steroid receptor is determined by its conformation upon ligand binding, as this structural change promotes or prevents its interaction with co-regulators (Beato et al., 1996). For transactivation, co-activators and components of the basal transcription machinery are recruited to the promoters of target genes to activate transcription, while co-repressors are recruited during transrepression to repress transcription (Beato et al., 1996; Hager et al., 2009).

In this thesis we will focus on the transactivation of gene expression via the AR and how it is influenced by the PR isoforms. In general, all steroid receptors, except the ER, activate transcription by binding to a classical steroid response element with the palindromic sequence GGTACAnnnTGTTCT (Beato, 1989). This sequence is termed a progesterone response element (PRE) for the PR or classical androgen response element (ARE) for the AR (Schauwaers et al., 2007; Africander et al., 2014). Additionally, the AR also recognizes a direct repeat sequence (GGCTCTTTCAGTTC) which has been termed the AR-selective ARE, since it was not activated by the GR (Sui et al., 1999; Claessens and Gewirth, 2004; Schauwaers et al., 2007). The ER on the other hand, specifically recognizes the constrained, palindomic estrogen response element (ERE) sequence (AGGTCAgagTGACCT) (Belandia and Parker, 2000).

## 1.3 Steroid receptors: Key role players in the development of breast and prostate cancer

### *1.3.1 Estrogens and the ER subtypes*

Both the ERα and ERβ are involved in the development of the normal breast and prostate, as well as the cancerous breast and prostate (Horvath et al., 2001; Förster et al., 2002; Palmieri et al., 2002; Attia and Ederveen, 2012; Murphy and Leygue, 2012; Cheng et al., 2013; Omoto and Iwase, 2015). However, it is well-known that these two receptors display differential roles in the regulation of physiological responses (Palmieri et al., 2002; Attia and Ederveen, 2012; Cheng et al., 2013).

Both ERα and ERβ are present in the normal mammary gland (Murphy and Leygue, 2012). ERα is largely responsible for mammary gland development (Förster et al., 2002; Palmieri et al., 2002; Murphy and Leygue, 2012; Cheng et al., 2013), while ERβ mediates the later stages of mammary gland

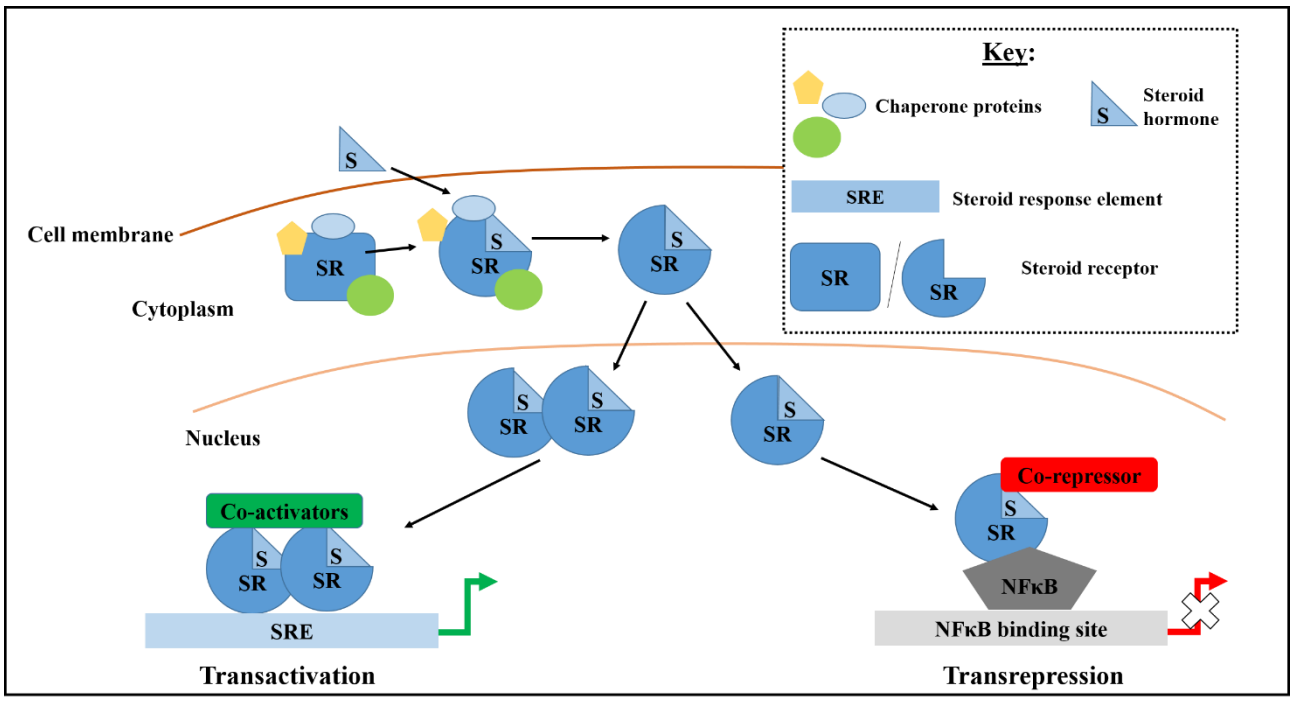


**Figure 1.2: An illustration of the general mechanism of action of steroid receptors.** A steroid hormone (S) diffuses across the cell membrane and binds to its cognate steroid receptor (SR), causing a conformational change in the receptor and the dissociation of chaperone proteins. The steroid-bound steroid receptor translocates to the nucleus where it can activate transcription (transactivation) by directly binding as a dimer to a steroid response element (SRE), or repress gene expression (transrepression) by tethering as a monomer to other DNA-bound transcription factors, such as NFκB. Figure adapted from: Africander et al., 2011.

differentiation and is the more abundantly expressed ER subtype (Förster et al., 2002). In breast cancer tissue, however, ERα levels are upregulated, while ERβ expression is decreased (Khan et al., 1994, 2002; Lawson et al., 1999; Zhao et al., 2003; Murphy and Leygue, 2012). Although both ERα and ERβ are also expressed in the developing prostate, and ERα is reported to be vital for normal prostate development, ERα is less abundant than ERβ in the normal adult prostate (Prins and Birch, 1997; Horvath et al., 2001; Prins et al., 2001a; Omoto et al., 2005). The reverse is true in prostate cancer, where ERα is expressed in about 60% of prostate cancer lesions and ERβ in less than 10% (Latil et al., 2001; Leav et al., 2001; Cheng et al., 2004; Yang et al., 2007; Bonkhoff and Berges, 2009; Megas et al., 2015). The high expression of ERα is associated with higher tumour grade, while the decrease in ERβ expression promotes changes in cell morphology leading to increased tumour proliferation (Horvath et al., 2001; Latil et al., 2001; Leav et al., 2001; Pasquali et al., 2001; Royuela et al., 2001; Fixemer et al., 2003; Zhao et al., 2003; Cheng et al., 2004). Taken together, the increases in ERα expression and decreases in ERβ expression in both breast and prostate cancer, suggests that ERα ultimately sustains tumour growth.

It is well-known that estrogens and the ER are key role players in breast cancer development (Ali and Coombes, 2000; Turner et al., 2017). For example, when bound to the most biologically active

estrogen, 17β-estradiol ($E_2$), ERα mediates proliferation of breast cancer cell lines by increasing the expression of cell cycle regulators such as *p21* and *cyclin D1*, as well as migration by decreasing the expression of cell adhesion proteins (Castro-Rivera et al., 2001; Liu et al., 2002; Alao, 2007; Liao et al., 2014). The role of ERβ, however, is dependent on the absence and presence of ERα. For example, although numerous studies have shown that ERβ is anti-proliferative in cells expressing ERα (Castro-Rivera et al., 2001; Liu et al., 2002; Alao, 2007), it is reported to be proliferative in ERα-negative breast cancer cells (Pettersson et al., 2000; Liu et al., 2002; Matthews and Gustafsson, 2003). It has been proposed that ERβ inhibits ERα-mediated transcription due to the formation of ERα and ERβ heterodimers (Pettersson et al., 2000; Liu et al., 2002; Lindberg et al., 2003; Paruthiyil et al., 2004; Murphy and Leygue, 2012). The roles of ERα and ERβ have been the topic of numerous reviews (Ali and Coombes, 2000; Gross and Yee, 2002; Palmieri et al., 2002; Matthews and Gustafsson, 2003; Mohamed et al., 2013), while similar reviews on their roles in prostate cancer are limited. We will thus focus mainly on the role of ERα and ERβ in prostate cancer for the rest of this section.

From the above studies, it appears that ERα and ERβ have similar roles in breast and prostate cancer (Horvath et al., 2001; Attia and Ederveen, 2012; Omoto and Iwase, 2015). The functions of these receptors in prostate cancer have been highlighted by studies in mice with prostate cancer tumours in which either ER subtype was knocked down (Ricke et al., 2007). For example, a study using ERα knockout mice (αERKO) and ERβ knockout mice (βERKO) demonstrated that the αERKO mice prostates showed tumour regression, suggesting that ERα mediates oncogenic effects in the prostate, while the findings with the βERKO mice showed that ERβ prevents prostate cancer progression (Ricke et al., 2007). In addition, ERα has been shown to mediate various oncogenic functions in prostate cancer by increasing the expression of the *pS2* gene, a well-studied ERE-containing gene in breast cancer, known to be associated with increased cell adhesion, migration and invasion (Kim et al., 2000). As in breast cancer, the role of ERβ in prostate cancer is dependent on the absence or presence of ERα. For example, while the expression of ERβ is considered to be anti-oncogenic in prostate cancer expressing ERα (Bonkhoff and Berges, 2009), it has been reported to mediate oncogenic effects in ERα-negative prostate cancer (Barkhem et al., 1998; Shazer et al., 2006). In addition, it has been shown that the growth of androgen-dependent and androgen-independent prostate cancer xenografts expressing only ERβ is inhibited in the presence of a pure ERβ antagonist (Barkhem et al., 1998; Shazer et al., 2006).

Numerous other studies have indicated the divergent roles of ERα and ERβ when co-expressed in prostate cancer. Most studies show that ERα stimulates prostate cancer cell proliferation, while ERβ inhibits ERα-mediated cell proliferation (vom Saal et al., 1997; McLachlan et al., 1998; Strauss et al., 1998; Prins et al., 2001a, 2001b, 2006, 2007; Attia and Ederveen, 2012). However, differential effects

are not limited to proliferation. For example, it has been shown that the occurrence of the *TMPRSS:ERG* gene fusion in prostate cancer is increased in the presence of the ERα-specific agonist proprylpyrazole triol, but decreased in the presence of the ERβ-specific agonist diarylpropionitrile (Setlur et al., 2008). The *TMPRSS:ERG* gene is a fusion of the *TMPRSS2* and E26 transformation-specific (ETS)-regulated *(ERG)* genes that occurs in 60% of prostate cancer tumours and ultimately leads to increases in prostate cancer cell proliferation and invasion (Setlur et al., 2008). In addition, while ERα mediated increases in cell invasion and proliferation, but decreases in apoptosis in the DU-145 prostatic carcinoma cell line, these effects were inhibited by ERβ (Cheng et al., 2004).

#### *1.3.1.1 Targeting the ER in breast and prostate cancer*

Breast cancer treatments focus on preventing the activation of the ER either by blocking the cytochrome P450 aromatase enzyme (CYP19A1) required for the biosynthesis of estrogen with aromatase inhibitors, or by blocking the ER using ER antagonists or selective ER modulators (SERMs), or by degrading the ER protein with selective ER downregulators (SERDs) (reviewed in Rau et al., 2005; Nagaraj and Ma, 2015). For example, although the SERM, tamoxifen, binds to the ER and allows the tamoxifen-bound ER to translocate to the nucleus, the ER cannot activate genes required for estrogen-mediated cell proliferation (Piccart et al., 2003). In contrast, the SERD, fulvestrant, downregulates the ERα protein thereby decreasing ERα activation by estrogens (Osborne et al., 2004; Agrawal et al., 2016). However, many women develop resistance to these treatments (Rau et al., 2005; Nagaraj and Ma, 2015), and thus current research is aimed at the development of improved therapies, such as compounds that have both ERα-selective antagonist and ERβ-selective agonist activity (Visser et al., 2013).

Although estrogens have been implicated in prostate cancer, these hormones have paradoxically been used in clinical trials of prostate cancer as a hypothalamus-pituitary-adrenal (HPA)-axis suppressor to decrease androgen production. Notably, the synthetic estrogen, diethylstilbestrol (DES) has formed part of certain ADT regimes, although it was not well tolerated (Citrin et al., 1991; Nelles et al., 2011). In addition, studies testing the use of 2-methoxyestradiol in castration-resistant prostate cancer (CRPC) patients have achieved mixed results. One study found that although the drug was well-tolerated, it did not confer any significant clinical benefits, while another study observed between a 20-40% decrease in the levels of the marker of prostate cancer, prostate-specific antigen (PSA) (Sweeney et al., 2005; Harrison et al., 2011).

Multiple SERMs have also been investigated for the treatment of prostate cancer (Bergan et al., 1999; Stein et al., 2001; Hamilton et al., 2003; Lissoni et al., 2005; Price et al., 2006). For example, 20% of CRPC patients treated with tamoxifen and 27% of androgen-independent prostate cancer patients

treated with raloxifene showed tumour regression after treatment (Bergan et al., 1999; Shazer et al., 2006). In addition, tamoxifen treatment caused a decline in PSA levels in 29% of patients in a phase II study of metastatic CRPC patients (Lissoni et al., 2005). Findings from clinical trials investigating prostate cancer treatment with the SERM toremifene, have shown contradictory results. One trial in androgen-independent prostate cancer patients showed that toremifene did not result in any clinical benefit (Stein et al., 2001), while a later clinical trial showed that the risk and incidence of prostate cancer development was decreased with toremifene treatment (Price et al., 2006). However, it should be noted that the Stein et al. (2001) investigation showing no clinical benefit consisted of a much smaller cohort of only 15 patients, while the trial indicating benefit had a cohort of 447 patients (Price et al., 2006).

In summary, although breast cancer treatments that typically target estrogen synthesis or the ER are mostly effective, resistance is known to develop in some patients. While the ER is also targeted in prostate cancer, findings from the few clinical studies are contradictory. It is thus evident that more studies are required to investigate ER targeted therapies in both breast and prostate cancer.

### *1.3.2 Androgens and the AR*

Androgens and the AR are vital for male physiology, and play a role in the development of the normal prostate and prostate cancer (Proverbs-Singh et al., 2015). The AR is expressed throughout the normal prostatic epithelium, and is upregulated during cancer progression when compared to the normal prostate (Latil et al., 2001; Qiu et al., 2008; Zeng et al., 2010; Barboro et al., 2014; Proverbs-Singh et al., 2015). In fact, high expression levels of the AR in prostate cancer are associated with disease progression and decreased disease-free survival (Lee et al., 2003).

Androgens acting via the AR are known to mediate prostate cancer progression via a number of mechanisms, such as increasing cell proliferation while limiting apoptosis (Azzouni and Mohler, 2012; Attard et al., 2016). One of the most well-studied mechanisms through which the AR induces proliferation, albeit indirectly, is via its control of the *TMPRSS2* gene (Robinson et al., 2015). Although the function of this gene is not well-described, it is known that 60% of prostate cancers exhibit chromosomal translocations leading to *TMPRSS2:ERG* gene fusion (Robinson et al., 2015). Induction of the *TMPRSS2* gene via the AR leads to increased ERG expression, and ERG in turn increases the expression of the oncogene, *c-myc*, which is associated with increased cell proliferation (Hoffman and Liebermann, 2008; Sun et al., 2008; Karantanos et al., 2013). A role for the AR in prostate cancer cell proliferation has been shown in various prostate cancer cell lines and mouse models in which cell proliferation was inhibited in the presence of the AR antagonist, bicalutamide,

or by AR silencing using siRNA (Colombel et al., 1993; Furuya et al., 1996; Gleave et al., 1999; Jayo et al., 2000; Yang et al., 2005; Arnold et al., 2007; Peters et al., 2011; Zhou et al., 2015; Komaragiri et al., 2016; Bae et al., 2017; Wang et al., 2017). Similarly, the importance of androgens are highlighted by studies showing that androgen signalling and androgen-mediated tumour proliferation are maintained in patients who become resistant to androgen deprivation therapy, the first-line treatment for advanced prostate cancer (Litvinov et al., 2003; Yang et al., 2005; Hoang et al., 2015).

Androgens acting via the AR have also been shown to decrease apoptosis. Apoptosis refers to the process of programmed cell death that occurs during normal cell development and ageing, and as a defence mechanism during cell damage, to maintain healthy cell populations in tissues (Elmore, 2007). In cancer however, cells develop mechanisms to evade apoptosis and thereby promote the survival of the cancerous cell (Elmore, 2007). It has been shown that treatment with the potent natural androgen 5α-dihydrotestosterone (DHT), acting via the AR, significantly decreased the expression of the apoptotic-promoting proteins, p53 and caspase-2, and increased the expression of the anti-apoptotic bcl-2 in several prostate cancer cell lines, including the LNCaP and VCaP cell lines (Colombel et al., 1993; Furuya et al., 1996; Gleave et al., 1999; Nantermet et al., 2004; Rokhlin et al., 2005; Komaragiri et al., 2016; Bae et al., 2017; Wang et al., 2017). Another study has indicated that DHT signalling via the AR prevents apoptosis by inhibiting a kinase pathway in the LNCaP cell line, which was reversed by treatment with bicalutamide and AR gene silencing (Lorenzo and Saatcioglu, 2008). In summary, it is evident that the AR contributes to prostate cancer by promoting prostate cancer cell growth and preventing apoptosis. Since the role of the AR in prostate cancer has been extensively reviewed, we will only focus on the AR in the context of breast cancer in the next section.

The role of androgens and the AR are not limited to prostate cancer, and have also been noted in the development of the normal and cancerous breast (Proverbs-Singh et al., 2015). The AR is abundantly expressed in the normal mammary epithelium (Hickey et al., 2012; Tarulli et al., 2014; Proverbs-Singh et al., 2015), while its expression in breast cancer varies depending on the breast cancer subtype. Specifically, about 25% of triple-negative breast cancer (TNBC) tumours express the AR (Micello et al., 2010; Niemeier et al., 2010; Park et al., 2010; Loibl et al., 2011; Wang et al., 2016), while it is expressed in 80% of ER-positive breast cancers (Agoff et al., 2003; Ogawa et al., 2008; Niemeier et al., 2010; Park et al., 2010; Loibl et al., 2011). The role of androgens and the AR in normal and cancerous breast development is complex. Normal breast growth is suppressed in prepubescent females who have high circulating androgens due to adrenal hyperplasia, suggesting an inhibitory role of androgens in breast cell proliferation (Forsbach et al., 2000). In support of this, numerous other studies have demonstrated inhibitory effects of androgens and the AR in breast

development of pubescent and adult females (Dürnberger and Kratochwil, 1980; Pashko et al., 1981; Casey and Wilson, 1984; Jayo et al., 2000; Dimitrakakis et al., 2003; Peters et al., 2011; Cheng et al., 2013). In breast cancer, however, the role of the AR seems to change depending on the presence of ERα. TNBC patients expressing the AR showed improved survival upon treatment with the AR antagonist, enzalutamide, suggesting that the AR mediates tumorigenic effects in the context of TNBC (Hickey et al., 2012; Lehmann and Pietenpol, 2014; Lim et al., 2014; McNamara et al., 2014; Barton et al., 2015; Lyons and Traina, 2017). In agreement with this study showing an oncogenic role for the AR in TNBC, proliferation studies in the $ER^-/AR^+$ MDA-MB-453 TNBC cell line showed that DHT-induced proliferation was inhibited by the AR antagonists hydroxyflutamide and enzalutamide (Birrell et al., 1995a; Cochrane et al., 2014). Moreover, the gene signature of this cell line has been shown to be similar to that of ER-positive breast cancer cell lines, such as the T47D and MCF-7 cell lines (Cochrane et al., 2014). This phenomenon is due to the AR activating similar gene sets as the oncogenic ERα (Cochrane et al., 2014), and suggests that the AR mimics the activity of ERα in ERα-negative cancers. Indeed, it has been shown that the activated AR, like activated ERα, can bind to the ERE (Peters et al., 2009).

In contrast to the studies showing that the AR may mediate oncogenic effects in AR-positive TNBC, experimental studies investigating the role of the AR in ERα-positive breast cancer cell lines mostly suggest that the AR protects against cell proliferation (Poulin et al., 1988; Birrell et al., 1995a; Szelei et al., 1997; Ortmann et al., 2002; Macedo et al., 2006; Cops et al., 2008). For example, numerous studies have shown that proliferation of the $ER^+/AR^+$ positive MCF-7, T47D and ZR-75-1 breast cancer cell lines is decreased in the presence of the synthetic androgen mibolerone (Mib), as well as the natural androgens DHT and testosterone (Poulin et al., 1988; Birrell et al., 1995a; Szelei et al., 1997; Ortmann et al., 2002; Macedo et al., 2006; Cops et al., 2008). In addition, treatment with DHT has been shown to promote apoptosis in the MCF-7, T47D and ZR-75-1 breast cancer cell lines by suppressing the expression of the anti-apoptotic *bcl-2* gene (Kandouz et al., 1999; Lapointe et al., 1999; Macedo et al., 2006). However, as previously mentioned at least one study has shown that treatment with DHT and Mib increased MCF-7 cell proliferation via the AR (Birrell et al., 1995a).

Finally, a protective role of the AR in ER-positive breast cancer progression has also been shown by a comparative study between AR knockout (ARKO) female mice and AR wild-type female mice (Simanainen et al., 2012). This study showed that the ARKO mice developed mammary tumours much sooner than wild-type mice (Simanainen et al., 2012). In contrast to this study, other studies have suggested that the AR may promote the growth of ER-positive breast cancer cell lines as well as ER-positive tumours (De Amicis et al., 2010; Wellberg et al., 2017). For example, AR overexpression in the ER-positive MCF-7 cell line has been shown to abrogate tamoxifen-mediated

repression of cell proliferation and increased anchorage-independent growth (De Amicis et al., 2010). Involvement of the AR in these abrogating-effects was confirmed when the effects were reversed in the presence of the AR antagonist, bicalutamide (De Amicis et al., 2010).

Taken together, the data suggests that the AR mediates proliferation and maintains tumour function in prostate cancer and TNBC. While most studies suggest that the AR may be protective against ERα-mediated oncogenicity, some studies have suggested that the AR may in fact not be protective in this context, as it can also mediate increases in proliferation. More studies are thus required to elucidate the precise role of the AR in ERα-positive breast cancer.

#### *1.3.2.1 Targeting the AR in breast and prostate cancer*

Therapies currently available for prostate cancer target androgen biosynthesis or the AR to prevent tumour progression. In addition, androgen deprivation therapy is used to treat patients with advanced or metastatic prostate cancer (Harris et al., 2009), and is often administered in combination with the AR antagonists flutamide or bicalutamide (Harris et al., 2009). Antagonists inhibit the activation of AR by preventing nuclear translocation (Brogden and Chrisp, 1991; Masiello et al., 2002; Rau et al., 2005; Osguthorpe and Hagler, 2011). In response to this treatment, patients initially show a significant decline in circulating testosterone and levels of the prostate cancer marker, PSA, indicating clinical and biochemical remission (Zlotta and Debruyne, 2005; Ross, 2008). However, this treatment is only temporarily effective and almost all patients develop CRPC, which is a far more aggressive and fatal malignancy (Ross, 2008; Azzouni and Mohler, 2012). Another AR antagonist, enzalutamide, has shown promising results in several clinical trials of CRPC patients, as it significantly reduced PSA levels, prolonged survival and improved quality of life in these patients (Scher et al., 2012; Bhattacharya et al., 2015; James et al., 2016; Kim et al., 2016). Finally, abiraterone acetate, the inhibitor of the enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17A1) which is essential for androgen biosynthesis, has been shown to reduce PSA levels by 50% and significantly improve survival in phase I and II clinical trials (Scher et al., 2008; Ryan et al., 2015; Poon et al., 2016). However, as abiraterone acetate also prevents the endogenous production of glucocorticoids, patients receiving this treatment are additionally administered synthetic glucocorticoids (Auchus et al., 2014).

Various clinical trials have also investigated the AR as a target for treatment of both AR-positive TNBC and ER-positive breast cancers, using both AR agonists and antagonists (reviewed in Anestis et al., 2015). For example, findings from a trial investigating treatment of AR-positive TNBC with the AR antagonist, bicalutamide, indicate that 20% of patients achieved complete tumour regression after therapy (Gucalp et al., 2013), suggesting that bicalutamide provides significant clinical benefit

for TNBC. Post-menopausal ER-positive breast cancer patients treated with a synthetic, non-metabolisable AR agonist, fluoxymesterone, in combination with the SERM, tamoxifen, have also shown clinical benefit as this combination treatment improved remission rates when compared to treatment with tamoxifen alone (Tormey et al., 1983; Ingle et al., 1991). In contrast, current phase II clinical studies investigating the use of the AR antagonist, enzalutamide, or the CYP17A1 inhibitor, abiraterone acetate, in combination with the aromatase inhibitor, exemestane, have thus far not shown any clinical benefit when compared to treatment with exemestane alone (O'Shaughnessy et al., 2014; Schwartzberg et al., 2014).

Medroxyprogesterone acetate (MPA) is a synthetic progestin that was designed to mimic the activity of the natural progesterone ($P_4$) via the PR (Croxatto, 2005). Although low doses of MPA used in hormone therapy (HT) are associated with increased breast cancer risk, higher doses have been effectively used in the treatment of advanced ER-positive breast cancer before or after tamoxifen failure (Focan et al., 2004; Zaucha et al., 2004). In addition, clinical studies have shown that AR expression is required for the response to MPA treatment after tamoxifen (Birrell et al., 1995b; Bentel et al., 1999; Buchanan et al., 2005). This is not surprising as MPA is known to elicit potent agonist activity via the AR (Africander et al., 2014; Louw-du Toit et al., 2017), suggesting that it is the androgenic properties of MPA that confer these clinical benefits in advanced breast cancer (Birrell et al., 1995b; Bentel et al., 1999; Buchanan et al., 2005; Carroll et al., 2017).

In summary, although resistance to prostate cancer therapy does often occur, it is clear that targeting AR activity is sufficient to limit prostate cancer progression in the short-term. In breast cancer, however, results from clinical studies targeting the AR are more complex, and indicate that more studies are required to elucidate the intricacies of AR signalling in breast cancer.

### *1.3.3 Progestogens and the PR*

Progestogens are a group of PR ligands, that include natural $P_4$ as well as progestins, which are synthetic compounds designed to mimic the actions of $P_4$ via the PR (Campagnoli et al., 2005; Sitruk-Ware, 2008). Progestins are widely used in menopausal HT and in contraceptives (Campagnoli et al., 2005; Sitruk-Ware, 2008), and have been linked to increased risk of breast cancer (Beral and Million Women Study Collaborators, 2003; Chlebowski et al., 2013, 2009, 2003; Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Fabre et al., 2007; Hunter et al., 2010; Li et al., 2012; Santen, 2014). Interestingly, the link between the progestin MPA and breast cancer was shown more than three decades ago when 80% of BALB/c mice developed mammary tumours weeks after treatment with MPA (Lanari et al., 1986). In contrast, most studies have suggested that $P_4$ does not

influence breast cancer risk (Carroll et al., 2017; Fournier et al., 2008, 2005; Lieberman and Curtis, 2017).

Until recently, the PR was considered as only a marker of ER functionality in breast cancer, since the ER is known to regulate PR expression by binding to an ERE in the promoter of the *PR* gene (Peters et al., 2009; Ravdin et al., 1992). Although the PR is expressed in 60-70% of breast cancers (Allred et al., 2012; Pichon et al., 1996; Thorpe, 1988; Wenger et al., 1993), its exact role in breast cancer is still an area of ongoing research. However, some studies in metastatic breast cancer patients on tamoxifen therapy have suggested that PR expression may predict success of endocrine therapy and survival, suggesting that the PR is a positive prognostic factor in breast cancer (Bardou et al., 2003; Elledge et al., 2000; Fisher et al., 1988; Kurozumi et al., 2017; Mohsin et al., 2004; Pertschuk et al., 1990, 1988; Sato et al., 2016; Snell et al., 2017). In contrast to these studies, another study has suggested that the PR is a poor prognostic factor and may predict disease recurrence in invasive breast cancer patients (Onoda et al., 2015). Moreover, studies investigating the role of the PR in breast cancer rarely distinguish between the PR isoforms, PRA and PRB. Such studies are important as it is known that the ratio of PRA to PRB is dysregulated in breast cancer, and that these isoforms may activate similar or different gene sets (Hopp, 2004; Kariagina et al., 2008; Sartorius et al., 1994). For example, ~50% of the total $P_4$-regulated genes in the T47D breast cancer cell line are regulated by PRB, while ~30% are regulated by PRA and ~20% by both isoforms (Kariagina et al., 2008; Sartorius et al., 1994). As PRB is transcriptionally more active than PRA when bound to ligand, we will describe the effects of PRB before PRA in this thesis. In addition, a study in a bi-inducible T47D cell line in which either PRB or PRA was expressed showed that, while both isoforms upregulated cell cycle regulator proteins and promoted proliferation, PRB also upregulated the expression of genes associated with DNA replication, and PRA the expression of the apoptotic marker *bcl-2* (Richer et al., 2002). Consistent with the latter results, another study in the same cell line has shown that regulation of the *bcl-2* gene could be mediated by PRA or a PRB:PRA heterodimer, but not by PRB alone (Jacobsen et al., 2002). Taken together, these studies highlight the need for investigating the individual roles of PRB and PRA in breast cancer.

As already mentioned, PRA expression levels are increased relative to PRB in breast cancer (Hopp, 2004). The importance of PRA:PRB ratios was suggested by a clinical study which showed that increased PRA expression relative to PRB may lead to tamoxifen resistance (Hopp, 2004). Other studies have suggested that tumours with increased PRA:PRB expression are of a higher tumour grade and respond poorly to treatment when compared to those with less PRA (Bamberger et al., 2000; Graham et al., 1995). In contrast to these studies, it has been shown that mice with higher PRA:PRB ratios are responsive to treatment with the PR antagonist, mifepristone (RU486), which mediated its

anti-proliferative effects via PRA (Wargon et al., 2015a, 2011, 2009). The underlying mechanism for the success in treatment with RU486 is due to an increased interaction between the PR and a co-repressor at the promoters of the *cyclin D1* and *c-myc* genes, resulting in decreased growth of PRA-rich tumours (Wargon et al., 2015b). However, some studies have brought into question the idea that PRA levels are higher than PRB levels (reviewed in Daniel et al., 2011; Knutson and Lange, 2014). These studies suggest that the current methodologies used to measure PRB levels are inaccurate due to the fact that PRB is highly transcriptionally active in the breast and therefore undergoes rapid post-translational modification and higher rates of turnover than PRA (reviewed in Daniel et al., 2011; Knutson and Lange, 2014). Therefore, more data is required on the relative levels of the PR isoforms present in breast tumours, and new technologies are required to elucidate the absolute levels of PRB relative to PRA.

Studies examining PR expression levels in prostate cancer are scarce and, as observed in breast cancer, do not often discriminate between PRB and PRA. Some studies have indicated that little or no PR mRNA is present in the normal prostatic epithelium (Hiramatsu et al., 1996; Yu et al., 2015, 2013), while others have shown that both PRB and PRA mRNA are present (Brolin et al., 1992; Latil et al., 2001; Luetjens et al., 2006; Nowakowska et al., 2016). Interestingly, both PRB and PRA mRNA have also been reported in prostate cancer (Luetjens et al., 2006). While some studies have indicated that PR mRNA expression is decreased in prostate cancer and CRPC when compared to the normal prostate (Hiramatsu et al., 1996; Yu et al., 2015, 2013), other studies have shown that it is increased when compared to normal or benign prostatic hyperplasia tissue (Brolin et al., 1992; Latil et al., 2001; Luetjens et al., 2006; Nowakowska et al., 2016). High PR expression levels have been correlated with tumour progression and expression of the proliferation marker Ki67 (Bonkhoff et al., 2001; Grindstad et al., 2015), suggesting that the PR promotes proliferation in prostate cancer. In summary, although the data from the limited studies investigating PR expression levels in the prostate are contradictory, most of the available studies suggest that PR expression may be a poor prognostic factor in prostate cancer.

To the best of our knowledge, only five studies have examined the mechanistic role of the PR in prostate cancer (Yu et al., 2013, 2014, 2015; Detchokul et al., 2015; Nowakowska et al., 2016). Interestingly, three mechanistic studies by the same research group have shown that the unliganded PR isoforms decrease cell migration and invasion of androgen-independent and androgen-dependent prostate cancer cell lines, and promote differentiation of prostate stromal cells (Yu et al., 2013, 2014, 2015). In addition, Yu et al. (2013) showed that PRA and PRB regulated different genes in the presence of $P_4$. PRA influenced the expression of genes involved in angiogenesis, while PRB modulated genes involved in cell cycle progression (Yu et al., 2013). Collectively the studies by Yu

and co-workers suggest that the PR may decrease prostate cancer tumorigenesis (Yu et al., 2013, 2014, 2015) which is in contrast to clinical studies showing that high PR expression is associated with prostate cancer tumour progression and clinical failure (Bonkhoff et al., 2001; Grindstad et al., 2015). In support of these clinical findings, however, some mechanistic studies suggest an oncogenic role for the PR in prostate cancer. For example, a study investigating an LNCaP model which no longer responds to androgens (androgen-independent) and clinical samples of patients with CRPC has implicated the PR as a possible mediator of resistance to treatment (Detchokul et al., 2015). This study showed that PR expression and activity was significantly upregulated when compared to all other transcription factors in response to DHT, and that oncogenesis was maintained by the regulation of various AR-target genes. These results suggest that the PR may be mimicking the AR by regulating the expression of these AR-target genes. Interestingly, Nowakowska et al. (2016) have shown that PR mRNA expression is increased in an LNCaP95 cell line resistant to abiraterone acetate and enzalutamide when compared to non-resistant cell lines. A similar increase in PR expression was observed upon AR knockdown in wild-type LNCaP cells (Nowakowska et al., 2016), suggesting that the AR regulates PR expression in prostate cancer, and that in the absence of AR signalling, the PR may mediate resistance to abiraterone acetate or enzalutamide. Interestingly, the GR has previously been shown to mediate resistance to enzalutamide by a similar mechanism in the LNCaP cell line (Arora et al., 2013). Moreover, it has been suggested that the AR inhibitor, ailanthone, may provide a mechanism to overcome enzalutamide resistance in various prostate cancer cell lines due to its ability to also inhibit PR activity (He et al., 2016).

In summary, while the limited evidence regarding the role of the PR in prostate cancer is contradictory, the available studies have provided impetus for the investigation of the PR isoforms as a potential target in prostate cancer and CRPC (Bonkhoff et al., 2001; Detchokul et al., 2015; Grindstad et al., 2015; Nowakowska et al., 2016).

#### *1.3.3.1 Targeting the PR in breast and prostate cancer*

Studies targeting the PR in breast cancer using selective PR modulators (SPRMs) or PR antagonists have shown contradictory results. For example, studies have indicated that the PR antagonists, lonaprisan and telapristone acetate, as well as various SPRMs, namely EC312, EC313 and CDB-4124, may oppose progestogen-mediated effects by decreasing the expression of the anti-apoptotic gene, *bcl-2*, inhibiting colony formation of T47D cells, and decreasing the number of PR-positive tumours in rats (Busia et al., 2011; Wiehle et al., 2011; Nickisch et al., 2013; Lee et al., 2016; Nair et al., 2016). In contrast to these studies, the PR antagonist ulipristal acetate has been shown to increase the proliferation of the T47D cell line (Communal et al., 2012).

Clinical trials investigating PR ligands in breast cancer therapies have had limited success due to structural similarities between the PR, AR and GR (Meyer et al., 1990; Wagner et al., 1996; Leonhardt and Edwards, 2002). For example, a trial investigating the use of the PR antagonist, RU486, and the SPRM, onapristone, for the treatment of post-menopausal breast cancer showed that patients did not effectively respond to therapy, and that significant liver toxicity was observed (Perrault et al., 1996; Han et al., 2007; Jonat et al., 2013). Although another study showed that the SPRM, onapristone, was effective in decreasing tumour size in patients, the trial was ended due to patients exhibiting hepatoxicity (Robertson et al., 1999). It is important to note that the effects of SPRMs or PR antagonists may not be PR-specific (Andrieu et al., 2015), thus future studies should investigate the effects of these drugs on both GR- and AR-mediated action prior to clinical testing.

To the best of our knowledge, two clinical trials are currently investigating the PR as a target in prostate cancer (Trial number: NCT02049190) (Jayaram and Nowakowska, 2015). While one trial is investigating the use of onapristone in combination with abiraterone acetate to treat advanced CRPC and metastatic CRPC (Trial number: NCT02049190) (reviewed in Antonarakis et al., 2016), the other trial is examining the use of onapristone before administration of either abiraterone acetate or enzalutamide in CRPC patients (Jayaram and Nowakowska, 2015). Preliminary results from the latter trial indicate that onapristone effectively abrogates PR expression with minimal toxicity (Jayaram and Nowakowska, 2015). However, whether onapristone results in any significant clinical benefits in CRPC patients has yet to be determined, and the likelihood that it will have cross-reactivity with the GR in prostate cancer patients, as observed in breast cancer patients, cannot be excluded (Robertson et al., 1999).

### *1.3.4 Crosstalk between the ER, AR and PR in breast and prostate cancer*

#### *1.3.4.1 ER-AR crosstalk*

A number of studies have investigated crosstalk between ERα and the AR in breast cancer (Kumar et al., 1994; Panet-Raymond et al., 2000; Peters et al., 2009; D'Amato et al., 2016), with several studies indicating that the activity of ERα is inhibited by the AR (Kumar et al., 1994; Panet-Raymond et al., 2000; Peters et al., 2009; D'Amato et al., 2016). The mechanism for this inhibition has been attributed to the AR competing with ERα for binding to EREs (Peters et al., 2009) or by the AR increasing the expression of ERβ (Rizza et al., 2014). Indeed, it has been shown that the AR can decrease ERα-mediated activation of the ERE-containing *pS2* gene (Panet-Raymond et al., 2000) and the ERE-containing *PR* gene (Peters et al., 2009). In contrast, other studies have suggested that the AR may in fact be required for optimal $E_2$-mediated oncogenicity in ERα-positive breast cancer cells. For example, a study in the MCF-7 breast cancer cell line has suggested that a functional AR is required

for an optimal response to $E_2$ (Cochrane et al., 2014), while another study has shown that treatment with $E_2$ redirects AR binding sites such that the AR primarily recognises ERE sequences (D'Amato et al., 2016). These studies suggest that the AR may be a poor prognostic factor in ER-positive breast cancer.

Conversely, it has been shown that liganded ERα decreased AR-mediated activation on an ARE (Kumar et al., 1994; Panet-Raymond et al., 2000) via a direct interaction between these receptors (Panet-Raymond et al., 2000). Although this study reported that the AR and ERβ do not directly interact (Panet-Raymond et al., 2000), a study from our laboratory has previously shown that the liganded AR inhibits the transrepression, but not transactivation, function of ERβ via an ERE (Easter Ndlovu, MSc thesis). As mentioned earlier, it has been shown that the activated AR can increase ERβ gene expression in the MCF-7 and ZR751 breast cancer cell lines by binding to an ARE sequence in the ERβ promoter region (Rizza et al., 2014). The increased ERβ resulted in decreased cell proliferation (Rizza et al., 2014), which may be due to ERβ limiting ERα-mediated proliferation.

To the best of our knowledge, no studies have investigated crosstalk between the AR and ERα in prostate cancer, while at least two studies have investigated AR-ERβ crosstalk (Muthusamy et al., 2011; Grubisha and DeFranco, 2013). Interestingly, both these studies showed that ERβ, when activated by either androgen metabolites (Grubisha and DeFranco, 2013), or estrogenic metabolites of DHT (Muthusamy et al., 2011), opposes AR-mediated prostate cancer cell proliferation.

In summary, the data suggest that significant crosstalk exists between ERα and the AR in breast cancer, which may explain the divergent role of the AR in ERα-positive versus ERα-negative breast cancers. In contrast, crosstalk between the AR and ERβ in breast cancer is less well investigated, while it appears to have an important role in prostate cancer. Finally, unlike the numerous studies examining the interaction between the AR and ERα in breast cancer, not much is known about the interplay between these receptors in prostate cancer.

#### *1.3.4.2 ER-PR crosstalk*

Several studies have investigated crosstalk between ERα and the PR in breast cancer (Giulianelli et al., 2012; Daniel et al., 2015; Mohammed et al., 2015; Singhal et al., 2016). For example, it has been shown that, even though MPA does not bind to the ER subtypes, ERα is required for the PR-mediated effects of MPA on breast cancer cell proliferation (Giulianelli et al., 2012). This study also showed that ERα and the PR are co-recruited to the promoters of the oncogenes, *cyclin D1* and *c-myc*, thereby increasing their expression (Giulianelli et al., 2012). In addition, both PR isoforms could interact with ERα in the nuclei of human MPA-treated cells. Another study has shown that unliganded PRB

enhances ERα-regulated gene expression and breast cancer cell proliferation (Daniel et al., 2015). In contrast to the above-mentioned studies indicating that crosstalk between ERα and the PR are associated with poor prognosis in breast cancer, two recent studies suggest that it is associated with a good prognosis when the PR is activated (Mohammed et al., 2015; Singhal et al., 2016). For example, Mohammed and co-workers showed that PR activated by $P_4$ or the synthetic progestin promegestone (R5020), redirects ERα to new chromatin binding sites, thereby inhibiting ERα-mediated oncogenic effects (Mohammed et al., 2015; Singhal et al., 2016). A more indirect mechanism of crosstalk has been shown between ERα and PRB in the T47D cell line treated with R5020 (Migliaccio et al., 1998; Ballare et al., 2003). Specifically, it has been shown that PRB-mediated activation of a proliferation-promoting kinase pathway was dependent on the presence of ERα, and prevented by both PR and ER antagonists (Migliaccio et al., 1998; Ballare et al., 2003). Whether crosstalk between ERα and the PR in breast cancer is associated with poor or good prognosis thus appears to be largely dependent on the presence and type of ligand used to activate the PR. Moreover, whether similar crosstalk mechanisms exist between ERβ and the PR is not known. Finally, similar crosstalk mechanisms between the ER subtypes and the PR has to the best of our knowledge, not been investigated in prostate cancer.

#### *1.3.4.3 PR-AR crosstalk*

To the best of our knowledge, no study has directly investigated crosstalk between the PR and AR in breast or prostate cancer. However, possible PR-AR crosstalk may be inferred from the available data. Members of the KLK serine protease family are known to be regulated by steroids in breast and prostate cancer (Nelson et al., 1999; Lai et al., 2009), and the PSA protein is transcribed from the AR-regulated *KLK3* gene (Nelson et al., 1999; Lai et al., 2009). Most KLK proteins are responsible for cleaving proteins involved in invasion and metastasis (Clements et al., 2004; Paliouras et al., 2007). A study by Lai et al. (2009) investigated the regulation of *KLK4* gene expression in the T47D breast and LNCaP prostate cancer cell lines, and found that the *KLK4* gene was expressed only in the presence of the PR and AR in breast and prostate cancer cells, respectively. Notably, while this study did not investigate whether the AR was expressed in the T47D cell line or PR expression in the LNCaP cell line, it is known that these receptors are endogenously expressed in these cell lines (Horwitz et al., 2008; Detchokul et al., 2015). This is significant since the *KLK4* gene promoter, like the *PSA* gene, contains both a PRE and selective ARE sequence (Nelson et al., 1999; Lai et al., 2009), and it is known that both the AR and PR can regulate the expression of genes via a PRE/classical ARE (Beato, 1989). Lai and co-workers found that $P_4$ upregulated *KLK4* gene expression via the PR binding to the PRE in the T47D cell line. In the LNCaP cell line, however, the data was less straightforward. Although the synthetic androgen R1881 induced both *KLK4* and *PSA* gene

expression, treatment with bicalutamide only partially abrogated the expression of both genes. Although these results suggest that the AR is not the only steroid receptor mediating these effects, the authors concluded that the effects on *PSA*, but not *KLK4*, gene expression were AR-mediated. Furthermore, the study revealed that the AR DBD was not binding directly to the *KLK4* selective ARE, and that the *KLK4* promoter lacking the PRE sequence could not be activated by R1881, leading the authors to suggest that the AR regulates *KLK4* gene expression by tethering to AR co-regulators. An oversight by this study was the fact that the authors did not consider that *KLK4* gene expression in the LNCaP cell line may be mediated by the AR binding to the PRE sequence, as it is known that the AR can bind to a PRE/classical ARE sequence (Dubé et al., 1976; Cowan et al., 1977; Lai et al., 2009). Moreover, the authors did not consider the fact that R1881 is known to bind the PR with high affinity (Dubé et al., 1976; Cowan et al., 1977). Thus, it is possible that the PR alone, or both the PR and AR may be mediating the activation of the putative AR-regulated *KLK4* gene in the LNCaP cell line via the PRE. In addition, some studies have also implicated crosstalk between androgen and progesterone signalling pathways in prostate cancer. For example, resistance to abiraterone acetate in prostate cancer has been linked to an AR mutant (T878A) which is more responsive to $P_4$. This $P_4$-induced mutant AR was shown to activate the expression of various AR-target genes (Chen et al., 2015). However, this study did not investigate the possibility that $P_4$ may be mediating these actions via the PR, and not the AR, which is likely since $P_4$ is a potent PR agonist (Khan et al., 2013) that is known to display AR antagonist activity (Africander et al., 2014). Furthermore, recent evidence suggests that $P_4$ levels are significantly upregulated in patients undergoing treatment with abiraterone acetate or enzalutamide (McKay et al., 2017; Montgomery et al., 2017), which is of concern since high PR expression levels are also present in prostate cancer and CRPC patients.

More evidence implicating a potential role for PR-AR crosstalk in prostate cancer arises from a study investigating the role of the GR, which is structurally homologous to both the PR and AR (Gao et al., 2005). As mentioned earlier, the PR, AR and GR recognise the same hormone response element sequence. This sequence is termed the PRE when the PR is bound, the classical ARE when the AR is bound, or the glucocorticoid response element (GRE) when the GR is bound (Beato, 1989). Arora and co-workers showed that the GR was able to confer resistance to enzalutamide by binding to and activating selective AREs (Arora et al., 2013). The GR was able to directly modulate the expression of various AR-target genes and it was found that more than 80% of AR- or GR-regulated genes were modulated by the reciprocal receptor. Importantly, the authors state that the restoration of androgen signalling cannot solely be explained by the actions of the GR, as 43% of AR target genes containing GR binding sites within their promoters were not modulated by the GR. This suggests that another steroid receptor may be involved in mediating resistance to enzalutamide. As the AR and PR share

88% overall sequence homology, and the PR is known to be associated with tumour progression and resistance to treatment in prostate cancer (Marhefka et al., 2001), it is highly likely that the PR may be also be involved in conferring resistance to enzalutamide.

## 1.4 Conclusion

A multitude of similarities exist between breast and prostate cancer. While the role of the AR in prostate cancer is well defined, its role in breast cancer seems to be dependent on the expression of the ER. Although the AR may be protective in ER-positive breast cancer (Peters et al., 2009), studies have shown that the AR may be pro-tumorigenic in ER-negative breast cancer via a mechanism involving binding of the AR to EREs (Peters et al., 2009; Cochrane et al., 2014). The role of other steroid receptors such as the PR have been largely neglected in breast and prostate cancers. However, several studies have begun to address this in breast cancer, while similar studies in prostate cancer are limited. While the PR seems to be a negative or positive prognostic factor in breast cancer depending on whether it is unliganded or liganded, as well as the nature of the ligand bound (Hopp, 2004; Mote et al., 2015), the PR appears to be a poor prognostic factor in prostate cancer (Bonkhoff et al., 2001; Grindstad et al., 2015). Since it is known that the AR can bind to EREs in the promoter of the *PR* gene, and that others have shown that AR inhibition decreases PR expression in breast cancer and prostate cancer (Peters et al., 2009; Nowakowska et al., 2016; Wellberg et al., 2017), it is likely that the AR may regulate PR expression in both cancers. Thus, it is likely that the AR and PR are co-expressed in a subset of breast and prostate cancers. As crosstalk between the AR and ER (Panet-Raymond et al., 2000; Peters et al., 2009; Rizza et al., 2014), as well as between ERα and PR (Mohammed et al., 2015) has been shown in breast cancer, it raises the question whether similar crosstalk mechanisms exist in prostate cancer. Interestingly, crosstalk between the GR and AR has been shown in prostate cancer, where the GR mimics AR activity by regulating AR-target genes. Considering that the PR, AR and GR are structurally homologous and recognise the same DNA sequences, it is likely that the PR may also mimic AR activity. Taken together, the available evidence suggests that an interplay between the AR and the PR may be possible. Understanding crosstalk mechanisms in breast and prostate cancer is imperative to our understanding of the role of steroid receptors as mediators of breast and prostate development and progression, and may aid in the development of more effective therapies for these cancers.

## 1.5 Aims of the study

Evidence in the literature suggests that the AR and PR are co-expressed in a subset of both breast and prostate cancer tumours and that PR expression is upregulated in both cancers. Given that crosstalk between steroid receptors plays an integral role in breast cancer, and that overlapping mechanisms exist between breast and prostate cancer, the main aim of this study was to investigate the effects of PR isoforms on AR-mediated transactivation in breast and prostate cancer cell lines. As the AR is known to transactivate via the classical ARE/PRE, selective ARE and ERE, while the PR is only known to transactivate via the PRE/classical ARE, AR-mediated transactivation was investigated on all of these response elements. The AR- and PR-negative MDA-MB-231 and PC3 cell lines were employed as *in vitro* models of breast and prostate cancer, respectively.

The specific aims were as follows:

1. To validate the proposed experimental system.
2. To evaluate the effect of increasing concentrations of PRB or PRA, in the absence of PR ligands, on the transactivation function of the AR via the PRE/classical ARE, selective ARE and ERE.
3. To determine the effect of increasing concentrations of PRB or PRA, in the presence of PR ligands, on the transactivation function of the AR via the PRE/classical ARE, selective ARE and ERE.
4. To elucidate whether PRB and PRA, like the AR, can transactivate via the selective ARE and ERE.
5. To determine whether PRB or PRA, in the absence and presence of PR ligands, would modulate AR-mediated effects on breast and prostate cancer cell proliferation.

# Chapter 2

# Materials and Methods

## 2.1 Introduction

The test compounds used in this study included the natural androgen 5α-androstan-17β-ol-3-one (5α-dihydrotestosterone; DHT), the synthetic androgen 7α,17α-dimethyl-19-nortestosterone (mibolerone; Mib), the natural progestogen 4-pregnene-3,20-dione (progesterone; $P_4$); the progestins 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (promegestone; R5020) and 17α-hydroxy-6α-methyl-4-pregene-3,20-dione 17-aceate (medroxyprogesterone acetate; MPA). All the test compounds were obtained from Sigma-Aldrich, South Africa, prepared in absolute ethanol (EtOH) and stored at -20°C. For promoter-reporter studies, the compounds were added to serum- and phenol red-free Dulbecco's Modified Eagle's medium (DMEM) supplemented with 3.5 g/L glucose (Sigma-Aldrich, South Africa). For proliferation studies, the compounds were added to phenol red-free DMEM supplemented with 3.5 g/L glucose (Sigma-Aldrich, South Africa), 10% charcoal-stripped (Addendum A) fetal calf serum (CS-FCS) and 100 IU/mL penicillin and 100 µg/mL streptomycin (1% penicillin/streptomycin) (Sigma-Aldrich, South Africa). Compounds were diluted such that a final EtOH concentration of 0.2% v/v was obtained, thus 0.2% v/v EtOH was used for control incubations (without test compounds), and will hence be referred to as the vehicle control.

## 2.2 Plasmid DNA

### *2.2.1 Expression vectors*

The cDNA expression vectors for human PRA (pSG5-hPR-A) and PRB (pSG5-hPR-B) were a kind gift from Prof. E. Kalkhoven (Utrecht University Medical Centre, Netherlands) (Wissink and Kalkhoven, 1996), while the empty pSG5 expression vector was kindly provided by Prof. G. Mellgren (University of Bergen, Norway). The pTAT-PRE-E1b-luc promoter-reporter construct driven by the E1b promoter and containing two copies of the TAT-PRE was obtained from Prof. G. Jenster (Erasmus University of Rotterdam, Netherlands) (Jenster et al., 1997), while the human AR cDNA expression vector (pSG5-hAR) was provided by Prof. H. Klocker (Medical University of Innsbruck, Austria) (Shatkina et al., 2003). The previously described selective ARE luciferase promoter-reporter construct, 4x*SC*-ARE1.2, was a kind gift from Prof. F. Claessens (University of Leuven, Belgium) (Verrijdt et al., 1999). Plasmids expressing human ERα (pSG5-ERα) and human ERβ (pSG5-ERβ) were obtained from Prof. F. Gannon (European Molecular Biology Laboratory, Germany) (Flouriot et al., 2000), while the 2xERE-pS2-pGL3 luciferase promoter-reporter construct containing two copies of the ERE was obtained from Prof. B. Belandia (Institute for Biomedical Research, Spain) (Belandia and Parker, 2000). The cDNA expression vectors expressing human mineralocorticoid

receptor (pRS-hMR) and human GR (pRS-hGR) were kind gifts from Prof. R. Evans (Howard Hughes Medical Institute, USA) (Arriza et al., 1987).

## 2.3 Preparation and transformation of competent bacterial cells

*Escherichia coli* (*E. coli*) DH5α cells were made competent using the calcium chloride method and transformed using the heat shock method (Sambrook et al., 1989). Briefly, a single colony of the *E. coli* DH5α cells were inoculated into 25 mL Luria Bertani (LB) broth (Addendum A), and grown for 6 hours at 300 rpm at 37ºC. The cells were subsequently exposed to a cold-shock on ice for 10 minutes before collecting the cell pellet by centrifugation at 6000 rpm for 3 minutes in the Avanti® J-E centrifuge (Beckman Coulter Inc., USA). The cell pellet was resuspended in 10 mL cold 0.1 M $CaCl_2$ solution and incubated on ice for 20 minutes before centrifugation as described above. Finally, the cells were resuspended in 5 mL cold 0.1 M $CaCl_2$ containing 15% glycerol and stored at -80ºC until needed. For the transformation, 100 µL of the competent cells were thawed on ice for 10 minutes and 100 ng plasmid DNA added (Tang et al., 1994; Froger and Hall, 2007). Following an incubation on ice for 30 minutes, the cell/DNA solution was exposed to a 30 second heat shock at 42ºC and incubated on ice for 5 minutes. The cells transformed with the desired plasmids were subsequently grown in super optimal broth medium with catabolite repression (SOC) (Addendum A) at 37ºC for 1 hour at 300 rpm. The streak-plate method was used to plate the transformed cells onto agar plates containing 50 µg/mL ampicillin, and incubated at 37ºC for 16 hours. Ampicillin was used since all the plasmids used in this study contain an ampicillin-resistance gene.

### *2.3.1 Plasmid DNA extraction*

Plasmid DNA was extracted using the NucleoBond® Xtra Maxi Plasmid Preparation kit (Macherey-Nagel, USA) as per the manufacturer's instructions. Briefly, a starter culture of the transformed cells was prepared by inoculating a single colony into 5 mL LB broth containing 50 µg/mL ampicillin for selection, followed by an incubation at 37ºC for 8 hours at 300 rpm. Thereafter, 5 mL of the starter culture was added to 250 mL LB broth and incubated at 37ºC for 16 hours at 300 rpm to allow the cells to reach the exponential growth phase. The cell pellet was collected by centrifugation at 6000 x g for 10 minutes at 4ºC. The All reagents used were provided in the NucleoBond® Xtra Maxi Plasmid Preparation kit (Macherey-Nagel, USA), unless otherwise specified. Briefly, the bacterial cells were resuspended in lysis buffer using an optimised NaOH/sodium dodecyl sulfate (SDS) lysis method (Birnboim and Doly, 1979). After equilibration of the anion-exchange chromatography column, the lysate was loaded onto the column and cleared by gravitational flow. Following several

wash steps, the plasmid DNA bound to the column was eluted, then precipitated, and dissolved in Tris-EDTA (TE) buffer (Addendum A).

### *2.3.2 Restriction enzyme digest and agarose gel electrophoresis*

To confirm the integrity and size of the extracted plasmid DNA, the undigested DNA and restriction enzyme digests of the DNA were separated on a 1% agarose gel by electrophoresis at 100 V (Addendum B, Fig. S1). Briefly, the plasmid DNA concentrations, measured in ng/µL, were obtained using a NanoDrop™ MicroVolume spectrophotometer (ThermoFisher Scientific, South Africa) which was available at the Stellenbosch University Central Analytical Facility (CAF). For digestion of the plasmids, 200 ng of the plasmid DNA was incubated with 0.2 U of the specific enzyme and an appropriate concentration of the respective enzyme buffer at 37ºC for 1 hour. The undigested and digested plasmid DNA were diluted to a final concentration of 10 ng/µL using 6x orange DNA loading dye (ThermoFisher Scientific, South Africa), and loaded onto the agarose gel prior to electrophoresis.

## 2.4 Cell culture

The MDA-MB-231 human breast adenocarcinoma cell line was a kind gift from Prof. A. Edkins (Rhodes University, South Africa), while the COS-1 monkey kidney and HEK293 human embryonic kidney cells were obtained from the American Type Culture Collection (ATCC, USA). All the above-mentioned cell lines were maintained in DMEM containing phenol red and 4.5 g/L glucose (Sigma-Aldrich, South Africa) supplemented with 10% fetal calf serum (FCS) (The Scientific Group, South Africa) and 1% penicillin/streptomycin (Sigma-Aldrich, South Africa). The MDA-MB-231 cell line was additionally supplemented with 2 mM L-glutamine (Sigma-Aldrich, South Africa), and this medium will hence be referred to as complete DMEM for this cell line. The PC3 human prostate carcinoma cell line was kindly provided by Prof. A. Swart (Stellenbosch University, South Africa). These cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 4.5 g/L glucose (Sigma-Aldrich, South Africa) supplemented with 10% FCS (The Scientific Group, South Africa) and 1% penicillin/streptomycin (Sigma-Aldrich, South Africa), and will hence be referred to as complete RPMI medium for this cell line. All cell lines were maintained in 75 $cm^2$ culture flasks (Bio-Smart Scientific, South Africa) at 37ºC in an atmosphere of 90% humidity and 5% $CO_2$, and sub-cultivated with 2 mL 0.25% (w/v) trypsin-0.53 mM EDTA solution (Sigma Aldrich, South Africa) when the cells reached 80-90% confluency. The cell lines were regularly tested for mycoplasma infection using the Hoechst staining technique (Freshney, 2010) and only mycoplasma negative cell lines were used for experiments (Addendum B, Fig. S2). All experiments were

conducted within the first 25 to 30 passages after thawing of the MDA-MB-231 and PC3 cell lines, respectively.

## 2.5 Luciferase promoter-reporter assays

The MDA-MB-231 and PC3 cell lines were maintained as described in Section 2.4 and seeded into 6 cm dishes at a density of $0.8x10^6$ cells per dish in complete DMEM or RPMI medium, respectively. The next day, the cells were transiently transfected with 300 ng of either the empty vector (pSG5) or the expression vector for the human AR (pSG5-hAR) and 3000 ng of the promoter-reporter construct containing either the PRE (pTAT-PRE-E1b-luc), selective ARE (4x*SC*-ARE1.2) or ERE (2xERE-pS2-pGL3) sequence, in the absence and presence of 300 or 1500 ng of the expression vector(s) for PRA (pSG5-hPR-A) or PRB (pSG5-hPR-B). XtremeGENE HP DNA transfection reagent (Sigma-Aldrich, South Africa) was used for transfections as per the manufacturer's instructions. The pSG5 empty vector was used as a filler plasmid to ensure a constant amount of 4800 ng total DNA in all experiments, and to confirm that responses observed with ligand in the presence of transiently transfected steroid receptor were due to the transfected steroid receptor. After a 24 hour incubation period, the transiently transfected cells were seeded into 96-well plates at a density of $1x10^4$ cells per well (Fig. 2.1) using phenol red-free DMEM supplemented with 3.5 g/L glucose (Sigma-Aldrich, South Africa), 10% CS-FCS and 1% penicillin/streptomycin (Sigma-Aldrich, South Africa) and allowed to settle. The following day, the cells were treated for 24 hours with either the vehicle control or 1 nM ligand(s) prepared in serum- and phenol red-free DMEM supplemented with 3.5 g/L glucose. Following the incubation period, the cells were washed with 200 µL phosphate-buffered saline (PBS) solution, lysed with 25 µL passive lysis buffer (Addendum A) and stored at -20°C. The cell lysates were thawed and 5 µL used to measure the luciferase activity in relative light units (RLU) using the Promega luciferase assay system (Promega, USA) and the Veritas microplate luminometer (Turner Biosystems, USA). RLU values were normalised to the total protein concentration (mg/mL) of each lysate, determined using the Bradford protein assay method (Bradford, 1976). The data is represented as fold induction with the vehicle control is set as 1 and all other responses set relative to this. In experiments where the PR and AR are co-expressed, the fold induction of the AR in the presence of DHT is set as 100%, while responses obtained in the presence of the PR isoforms is expressed relative to that of the AR alone.

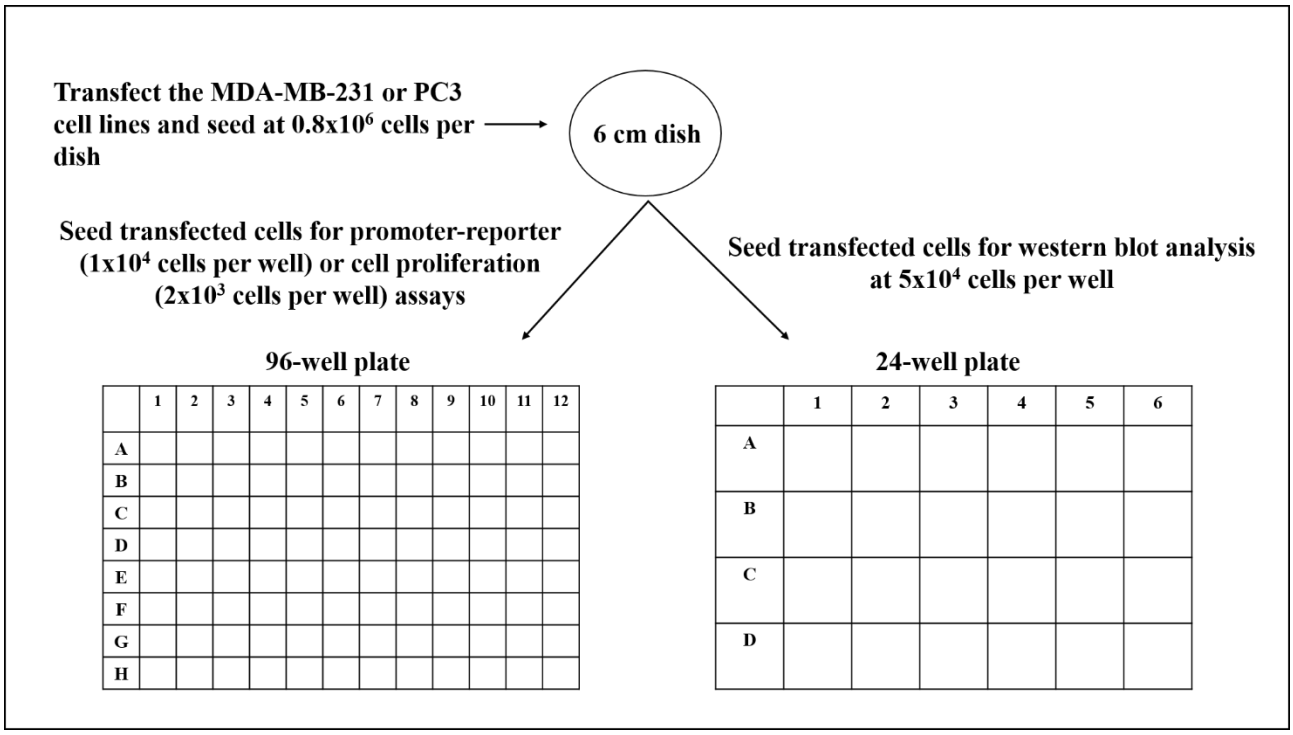


**Figure 2.1: Illustration of the experimental layout for promoter-reporter, cell proliferation and western blot assays.** MDA-MB-231 or PC3 cells were seeded into 6 cm dishes at $0.8x10^6$ cells per dish in complete DMEM or RPMI medium, respectively. The cells were subsequently transiently transfected with the appropriate expression vectors and incubated for 24 hours before seeding into either 96-well plates at $1x10^4$ cells per well for promoter-reporter or $2x10^3$ cells per well for cell proliferation assays, or 24-well plates at $5x10^4$ cells per well for western blot analysis.

## 2.6 Western blot analysis

### *2.6.1 Preparation of protein lysates*

To observe changes in AR, PRA, and PRB expression levels upon transfection, the MDA-MB-231 and PC3 cells were seeded into 24-well plates at a cell density of $5x10^4$ cells per well in phenol red-free DMEM supplemented with 3.5 g/L glucose (Sigma-Aldrich, South Africa), 10% CS-FCS and 1% penicillin/streptomycin (Sigma-Aldrich, South Africa) (Fig. 2.1). The following day, the cells were treated with the vehicle control which was prepared in serum- and phenol red-free DMEM supplemented with 3.5 g/L glucose (Sigma-Aldrich, South Africa), and incubated for a further 24 hours. Thereafter, the cells were rinsed with 500 µL PBS per well before lysis with 100 µL passive lysis buffer (Addendum A). To pellet the cell debris, the samples were centrifuged for 10 minutes at 12 000 x g at 4°C, and the protein lysate transferred to a sterile tube. Protein concentrations of the samples were determined using the Bradford protein determination method (Bradford, 1976). The amount of 2x Laemmli SDS-sample buffer that was added to the samples varied depending on the protein concentration.

To characterise the endogenous steroid receptor expression of the MDA-MB-231 and PC3 cell lines, these cells were maintained as described in Section 2.4 and seeded into 6-well plates at a density of $2.5x10^5$ cells per well and incubated until cells reached confluency. Positive controls were prepared by maintaining COS-1 and HEK293 cells as described in Section 2.4 and seeding these cells into 12-well plates at a density of $1x10^5$ cells per well. The COS-1 cells were transiently transfected with 250 ng of an expression vector encoding either the AR, ERα, ERβ or GR, while the HEK293 cells were similarly transiently transfected with PRA or PRB, using the XtremeGENE HP DNA transfection reagent (Sigma-Aldrich, South Africa) as per the manufacturer's instructions. Negative controls were prepared by seeding untransfected COS-1 cells into 12-well plates at a density of $1x10^5$ cells per well. All cells were grown until confluency was reached, washed with PBS solution and then lysed with 80 µL of 2x Laemmli SDS-sample buffer (Addendum A).

### *2.6.2 SDS-PAGE and western blot analysis*

Cell lysates were denatured by boiling at 95°C for 10 minutes. The proteins were separated on a 10% SDS-polyacrylamide gel at 100 V for 15 minutes, followed by 200 V for 1 hour in SDS-PAGE running buffer (Addendum A). The broad-range Color Prestained Protein ladder was used as a reference standard (New England BioLabs Inc., USA). The separated proteins were transferred onto nitrocellulose membranes (AEC Amersham, South Africa) in ice-cold transfer buffer (Addendum A) using the BioRad transfer unit (Bio-Rad, South Africa) for 1.5 hours at 180 mA. Thereafter, the nitrocellulose membranes were blocked for 2 hours in 10% w/v fat-free milk powder prepared in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature with agitation. The membranes were then washed with TBST to remove residual milk proteins, and subsequently incubated with the appropriate primary antibody for 16 hours with agitation at 4°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Following the incubation with primary antibody, the membranes were washed with TBST once for 15 minutes, twice for 5 minutes, and once in TBS for 5 minutes. The membranes were then incubated with the relevant horseradish peroxidase (HRP)-conjugated secondary antibody prepared in 10% w/v fat-free milk powder in TBST for 1 hour at room temperature with agitation. The membranes were subsequently washed as described above and the proteins were visualised using the Clarity™ Western ECL Substrate (Bio-Rad, South Africa) and the MyECL imaging system (Thermo Fisher Scientific, South Africa). The images were quantified using the myImageAnalysis software version 2.0 (Thermo Fisher Scientific, South Africa), and specific steroid receptor protein levels were normalised to that of GAPDH. The details of the primary and secondary antibodies used in this study are summarised in Table 2.1.

**Table 2.1 Details of primary and secondary antibodies used for western blotting.**

| Protein | Primary antibody | Dilution | Secondary antibody | Dilution |
|---|---|---|---|---|
| **AR** | AR-441 | 1:1000 | Goat anti-mouse | 1:3000 |
| **ERα** | ERα HC-20 | 1:1000 | Goat anti-rabbit | 1:1000 |
| **ERβ** | ERβ H-150* | 1:500 | Goat anti-rabbit | 1:1000 |
| **GR** | GR H-300 | 1:3000 | Goat anti-rabbit | 1:4000 |
| **PRA/B** | PGR-312** | 1:500 | Goat anti-mouse | 1:2000 |
| **GAPDH** | GAPDH 0411 | 1:3000 | Goat anti-mouse | 1:2000 |

All antibodies were purchased from Santa Cruz Biotechnology (USA), while antibodies purchased from Abcam (UK) are indicated by * and antibodies purchased from Leica Biosystems (UK) are indicated by **.

## 2.7 Cell proliferation assays

The MDA-MB-231 and PC3 cell lines were maintained as described in Section 2.4 before seeding into 6 cm dishes at a density of $0.8x10^6$ cells per dish in complete DMEM or RPMI medium, respectively. The next day, the cells were transiently transfected with 300 ng of either the empty vector (pSG5) or the expression vector for the human AR (pSG5-hAR), using the XtremeGENE HP DNA transfection reagent (Sigma-Aldrich, South Africa) as per the manufacturer's instructions. Following a 24 hour incubation, the transfected cells were seeded into 96-well plates at a density of $2x10^3$ cells per well (Fig. 2.1) and allowed to settle. The cells were then treated for 72 hours with either the vehicle control or 1 nM of the relevant ligand prepared in phenol red-free DMEM supplemented with 3.5 g/L glucose, 10% CS-FCS and 1% penicillin/streptomycin. Following the incubation, new compound mixtures were prepared and the cells were re-treated for a further 48 hours, before the addition of 50 µL of the colorimetric 3-(4,5-di dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma-Aldrich, South Africa) and 150 µL phenol red-free DMEM supplemented with 10% CS-FCS and 1% penicillin/streptomycin. MTT is based on the principle that the yellow MTT thiazolyl solution is reduced by NADPH-dependent oxidoreductase enzymes present in the mitochondria of metabolically active cells to form purple, insoluble formazan crystals that can be solubilised and quantified spectrophotometrically. Following the 4 hour incubation period with MTT, the solution was aspirated and 200 µL dimethyl sulphoxide (DMSO)

(Sigma-Aldrich, South Africa) added to solubilise the formazan crystals. The absorbance was measured at 540 nm using the BioTek spectrophotometer (BioTek Instruments, USA). For the alamar blue proliferation assay, following the initial 72 hour incubation with compounds, new compound mixtures were prepared and the cells were re-treated for a further 168 hours, before the addition of 20 µL of the resazurin sodium salt (Sigma-Aldrich, South Africa) to a final concentration of 0.15 mg/mL. The absorbance was read at 570 nm and 600 nm using the BioTek spectrophotometer (BioTek Instruments, USA). The alamar blue proliferation assay is a non-toxic alternative to the MTT assay, and is based on a similar principle in which resazurin sodium salt is reduced by active oxidoreductase enzymes in the mitochondria of living cells. For both the MTT and alamar blue proliferation assays, proliferation is shown as a fold induction obtained with the specific compound relative to the vehicle control set as 1.

## 2.8 Data manipulation and statistical analysis

GraphPad Prism® version 5.00 (GraphPad Software, USA) was used for graphical representation, data manipulation and statistical analysis. Data was presented as means ± standard error of the mean (SEM) of at least two independent experiments, unless otherwise stated. Column statistics were applied to analyse the grouped data using one-way analysis of variance (ANOVA) and Newman Keul's post-tests (to compare all columns). Statistically significant differences are indicated by * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). No statistical difference is indicated by ns where $p>0.05$.

# Chapter 3

# Results

## 3.1 Confirmation that MDA-MB-231 and PC3 cell lines do not express AR or PR

The MDA-MB-231 breast adenocarcinoma and the PC3 prostate adenocarcinoma cell lines were identified as model cell lines for this study, as they have both been reported to lack AR and PR expression (Sasaki et al., 2002; Terakawa et al., 2010; Holliday and Speirs, 2011; Narayanan et al., 2014). To confirm the absence of these steroid receptors, and investigate whether any other steroid receptors are expressed in the MDA-MB-231 and PC3 cell lines, we characterised endogenous steroid receptor expression by performing western blot analysis with lysates of the cell lines. The results in figure 3.1 indicate that only the GR is expressed in the MDA-MB-231 cell line, while only ERα is expressed in the PC3 cell line. Notably, the commercial antibody used to detect ERβ was not optimal as detection of ERβ in the positive control showed minimal expression of ERβ. Thus, we cannot definitively say whether or not the MDA-MB-231 and PC3 cell lines express endogenous ERβ.

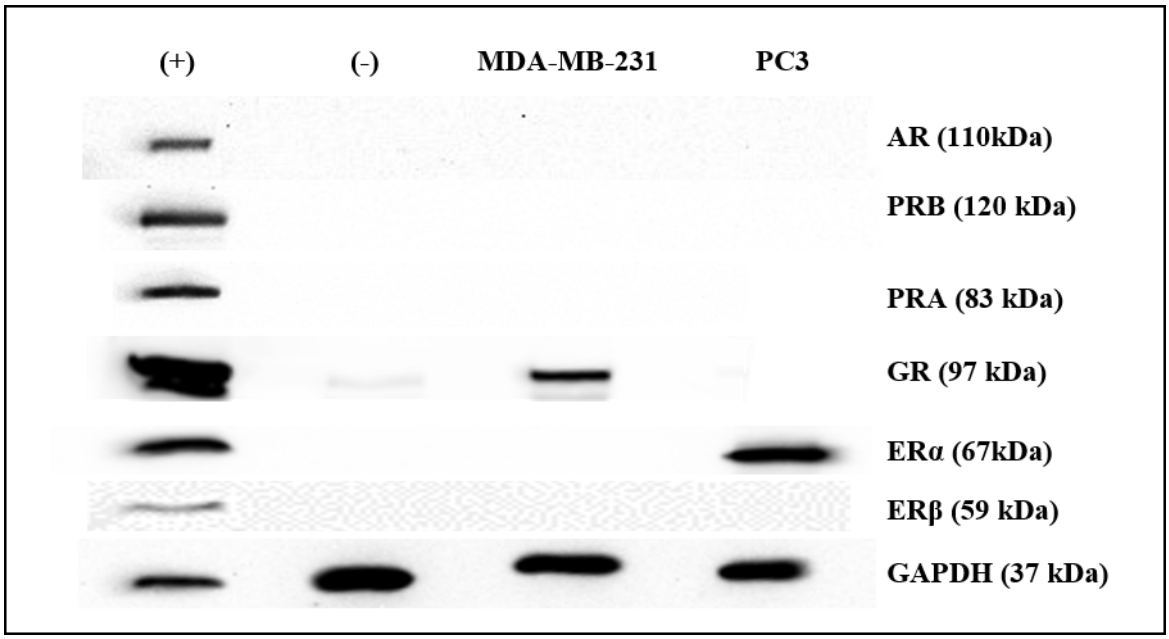


**Figure 3.1: Characterising the endogenous steroid receptor expression in MDA-MB-231 breast cancer and PC3 prostate cancer cell lines.** Whole cell extracts were prepared from the MDA-MB-231 and PC3 cell lines, and protein lysates analysed by western blotting using antibodies specific to the AR, PRA, PRB, GR, ERα, ERβ, or GAPDH (loading control). Protein lysates from COS-1 cells transfected with expression vectors encoding either the AR, GR, ERα or ERβ, or HEK293 cells transfected with expression vectors for either PRA or PRB were used as positive controls (+). Untransfected COS-1 cells were used as a negative control (-).

## 3.2 The transcriptional activity of PRB and the AR was confirmed on selected response elements

Given that we had confirmed that neither the MDA-MB-231 breast cancer nor PC3 prostate cancer cell lines express endogenous AR, PRA or PRB, these cell lines were employed to investigate the transcriptional activity of overexpressed AR and/or PRB on specific steroid response elements. PRB was used to validate the PRE as it is known to be more transcriptionally active than PRA in the presence of ligand (Kariagina et al., 2008). Since the DBD of steroid receptors is highly conserved, most steroid receptors can bind the same DNA response element (Gao et al., 2005). Thus, while PR isoforms can transactivate via a PRE, the AR, GR and MR can also recognize this response element. We thus used the PRE as a response element for PRB, but also as a response element for the AR (herein referred to as a classical ARE). Although the PRE/classical ARE and the response element for the ER, namely the ERE, are highly dissimilar, an ERE was also employed as Peters et al. (2009) had previously shown that the AR could bind to this response element. In addition, a previously described response element via which the AR but not the GR could transactivate, called an AR-selective ARE, was also used (Verrijdt et al., 1999). Thus, we wanted to validate the luciferase promoter-reporter assay in the MDA-MB-231 and PC3 cell lines using luciferase promoter-reporter constructs containing the PRE/classical ARE, selective ARE and ERE.

MDA-MB-231 breast cancer and PC3 prostate cancer cell lines were thus transiently transfected with either an empty vector (indicated throughout by grey bars) or PRB (indicated throughout by blue bars) together with the PRE luciferase (PRE-luc) promoter-reporter construct, and the cells treated with various progestogens (natural and synthetic PR ligands) (Fig. 3.2 A-B). Specifically, we used the classical potent synthetic progestin, R5020, a so-called PR-specific agonist, the natural PR ligand, $P_4$, and MPA, a synthetic PR ligand used clinically in HT and contraception in women, and which has also been investigated as a potential male contraceptive (Bain et al., 1980; Doody and Bain, 1985; Li et al., 2012). Similarly, these cell lines were transiently transfected with the empty vector or the AR (indicated throughout by white bars) and either the classical ARE-luc, selective ARE-luc or ERE-luc promoter-reporter constructs (Fig. 3.2 C-H). These cells were treated with the classical potent, non-metabolisable synthetic androgen Mib, the natural ligand for the AR, DHT, or MPA, which is known to elicit AR agonist activity similar to that of DHT (Africander et al., 2014).

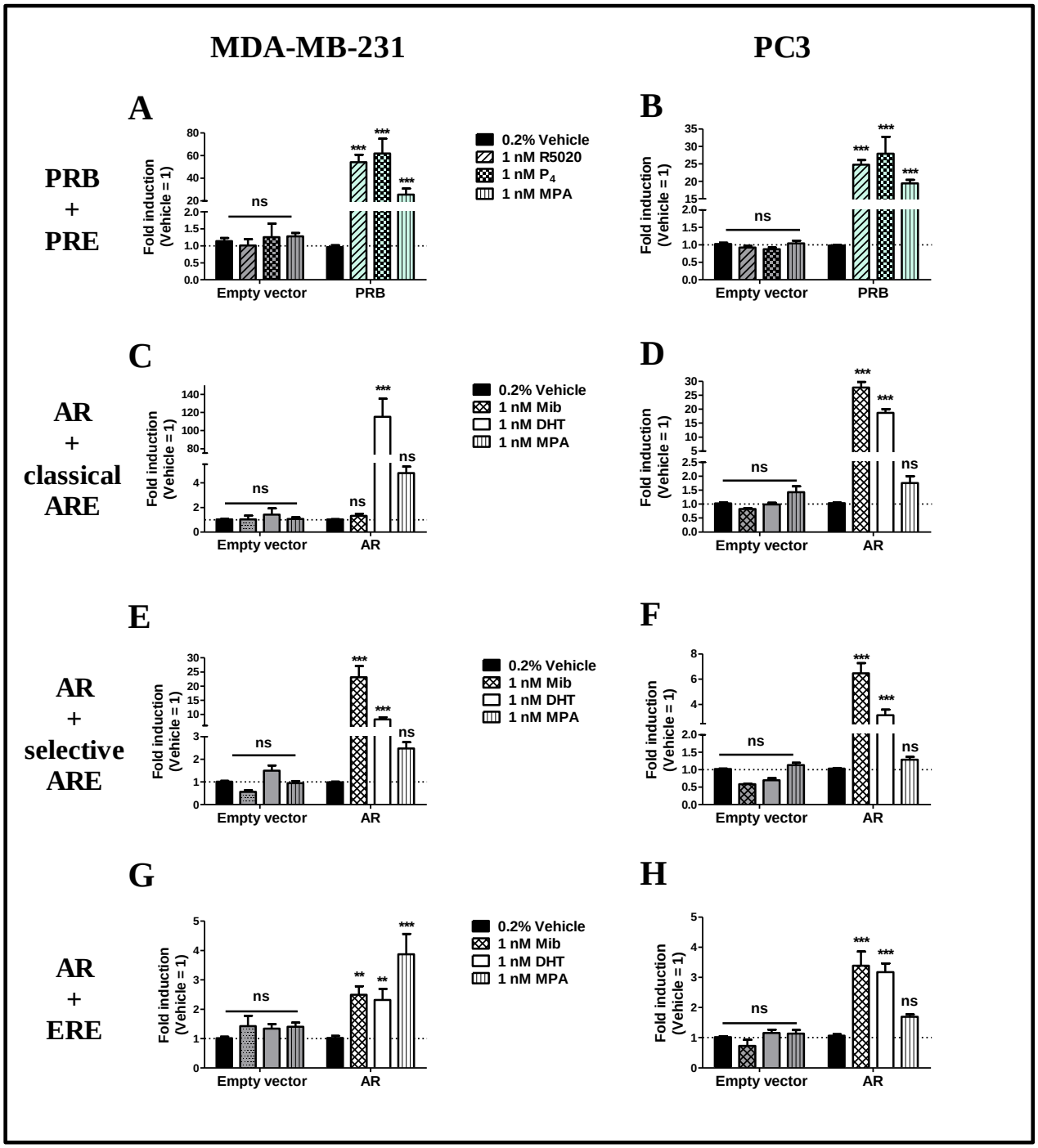


**Figure 3.2: PRB and AR are transcriptionally active in promoter-reporter transactivation assays in both the MDA-MB-231 and PC3 cell lines.** MDA-MB-231 breast cancer and PC3 prostate cancer cells were transiently transfected with 300 ng of the empty vector (pSG5) (grey bars; A-H) or pSG5-hPR-B (blue bars; A-B) and 3000 ng of the pTAT-PRE-E1b-luc reporter construct, or 300 ng of the pSG5-hAR (white bars; C-H) and 3000 ng of either the pTAT-PRE-E1b-luc (C-D), 4x*SC*-ARE1.2 (E-F) or the 2xERE-pS2-pGL3 reporter constructs (G-H). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction is shown with the vehicle control set as 1 and all other responses set relative to this. The results indicate the average of at least two independent experiments for all ligands except Mib (C), with each condition performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

Figure 3.2 A-B shows significant activation of the PRE-luc via PRB in the presence of R5020, $P_4$ and MPA in both the MDA-MB-231 and PC3 cell lines. Since no activation was observed in the absence of transfected receptor in either cell line, this confirms that the activation was due to the transfected PRB (Fig. 3.2 A-B). Significant activation of selective ARE by either Mib or DHT was also detected only in the presence of transfected AR in both cell lines (Fig. 3.2 E-F). In addition, DHT activated the classical ARE (Fig. 3.2 C-D) and ERE (Fig. 3.2 G-H) in both cell lines. The findings on the ERE are in line with the study by Peters et al. (2009) who showed that the AR could bind to the ERE. Surprisingly, although Mib also activated the ERE in both cell lines (Fig. 3.2 G-H), activation of the classical ARE by Mib was only observed in the PC3 cell line (Fig. 3.2 C-D). It should be noted that results for Mib on the classical ARE in the MDA-MB-231 cell line were from a single experiment (Fig. 3.2 C). Furthermore, MPA caused AR-mediated activation on the ERE in the MDA-MB-231 (Fig. 3.2 G), but not PC3 (Fig. 3.2 H), cell line. Taken together, these results suggest that PRB is required for progestogen-induced activation of the PRE, while the AR is required for activation of all three response elements by Mib and DHT, except in the case of Mib on the classical ARE in the MDA-MB-231 cell line.

### 3.3 Activation of PR isoforms by Mib and/or DHT, and the AR by R5020, are promoter- and/or cell line-dependent

It is known that the AR and PR share 55% DNA sequence identity within their LBD (Gao et al., 2005), and that MPA is a potent agonist for both receptors (Poulin et al., 1989; Africander et al., 2014), while the potent PR agonist, $P_4$, elicits very weak AR agonist activity on both the classical and selective AREs (Poulin et al., 1989; Africander et al., 2014). Thus, we next investigated whether the other AR and PR ligands (Mib, DHT and R5020) employed in this study could also elicit off-target effects via these receptors on the various response elements. Here, we included both PRB and PRA as the respective receptors were co-expressed with the AR in subsequent experiments. MDA-MB-231 and PC3 cell lines were transiently transfected with either the empty vector, PRB, PRA or the AR, and either the PRE/classical ARE-luc, selective ARE-luc or ERE-luc. Cells transfected with PRB (Fig. 3.3) or PRA (Fig. 3.4) were treated with Mib or DHT, while those transfected with the AR were treated with R5020 (Fig. 3.5).

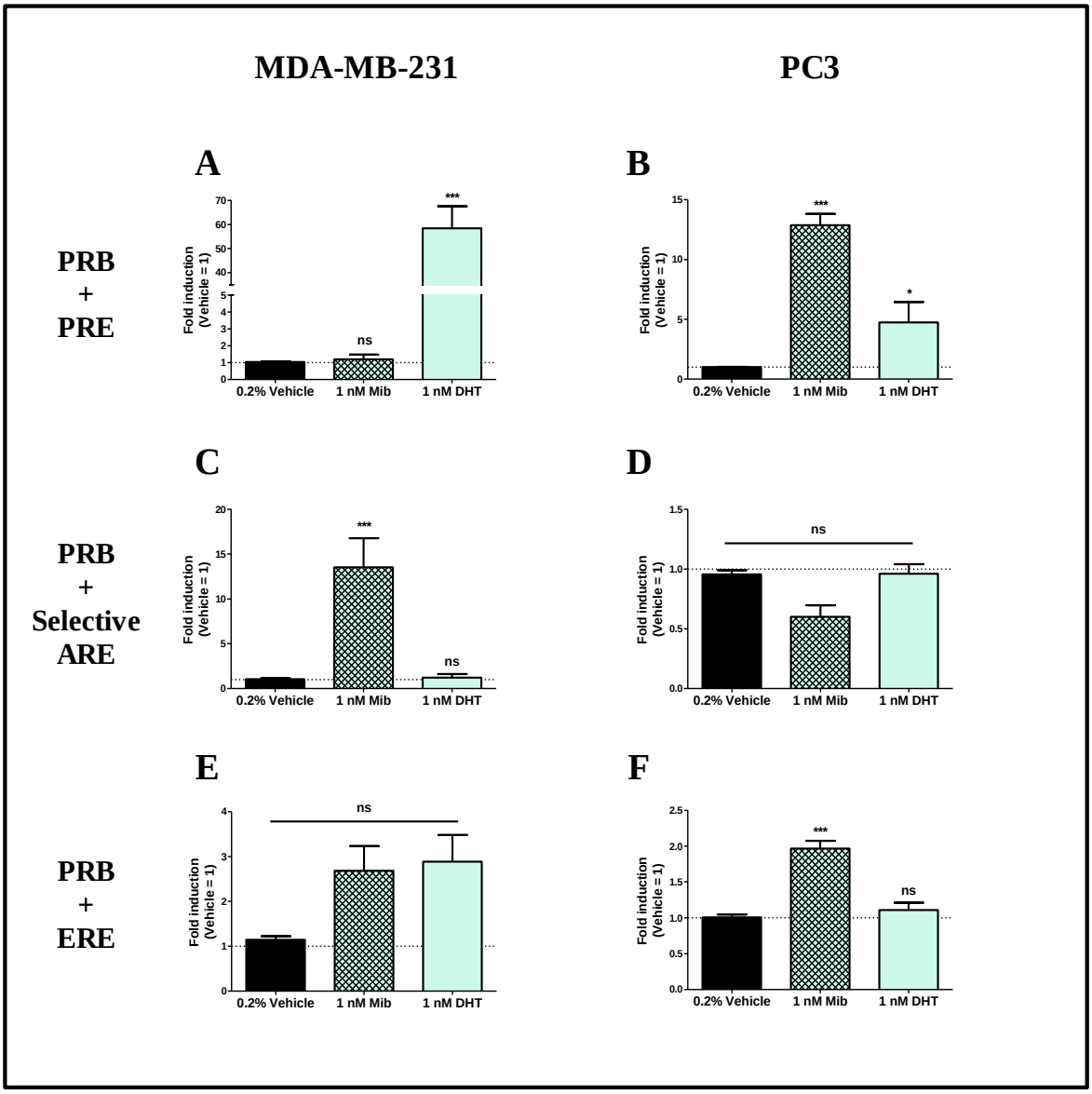


**Figure 3.3: Mib modulates PRB activity in a cell line- and promoter-specific manner, while DHT is a PR agonist only via PRE in both MDA-MB-231 and PC3 cells.** MDA-MB-231 breast cancer and PC3 prostate cancer cells were transiently transfected with 300 ng of pSG5-hPR-B (blue bars; A-F) and 3000 ng of the pTAT-PRE-E1b-luc (A-B), 4xSC-ARE1.2 (C-D) or the 2xERE-pS2-pGL3 reporter constructs (E-F). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM Mib or DHT for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction is shown with the vehicle control set as 1 and all other responses set relative to this. The results indicate the average of at least two independent experiments for all ligands except Mib (A), with each condition performed in triplicate (±SEM). Statistical analysis was performed using a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

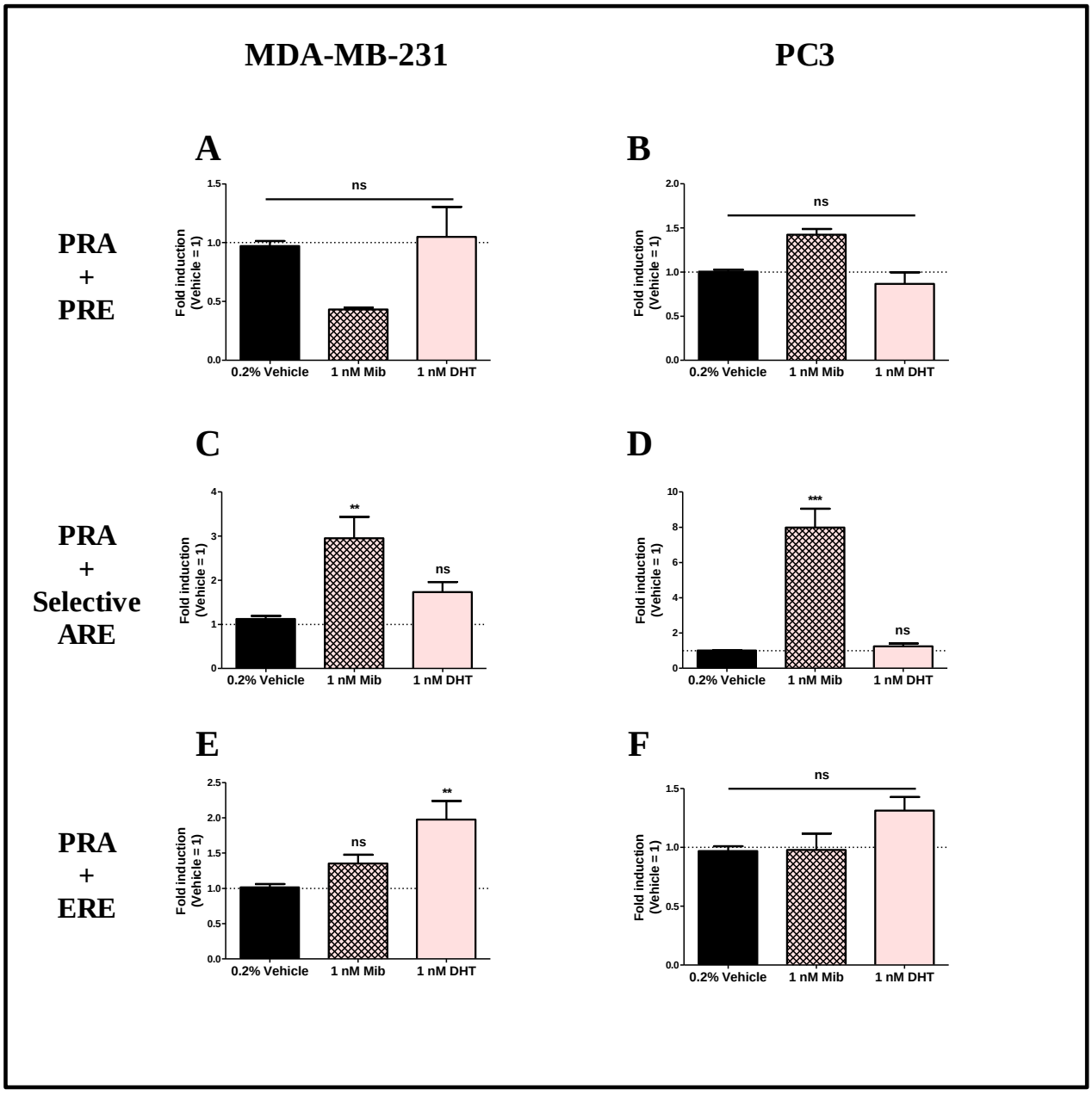


**Figure 3.4: Mib displays agonist activity via PRA only on the selective ARE in both cell lines, while DHT is only a PRA agonist via the ERE in the MDA-MB-231 cell line.** MDA-MB-231 breast cancer and PC3 prostate cancer cells were transiently transfected with 300 ng of pSG5-hPR-A (pink bars; A-F) and 3000 ng of the pTAT-PRE-E1b-luc (A-B), 4x*SC*-ARE1.2 (C-D) or the 2xERE-pS2-pGL3 reporter constructs (E-F). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM Mib or DHT for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction is shown with the vehicle control set as 1 and all other responses set relative to this. The results indicate the average of at least two independent experiments for all ligands except Mib (A), with each condition performed in triplicate (±SEM). Statistical analysis was performed using a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

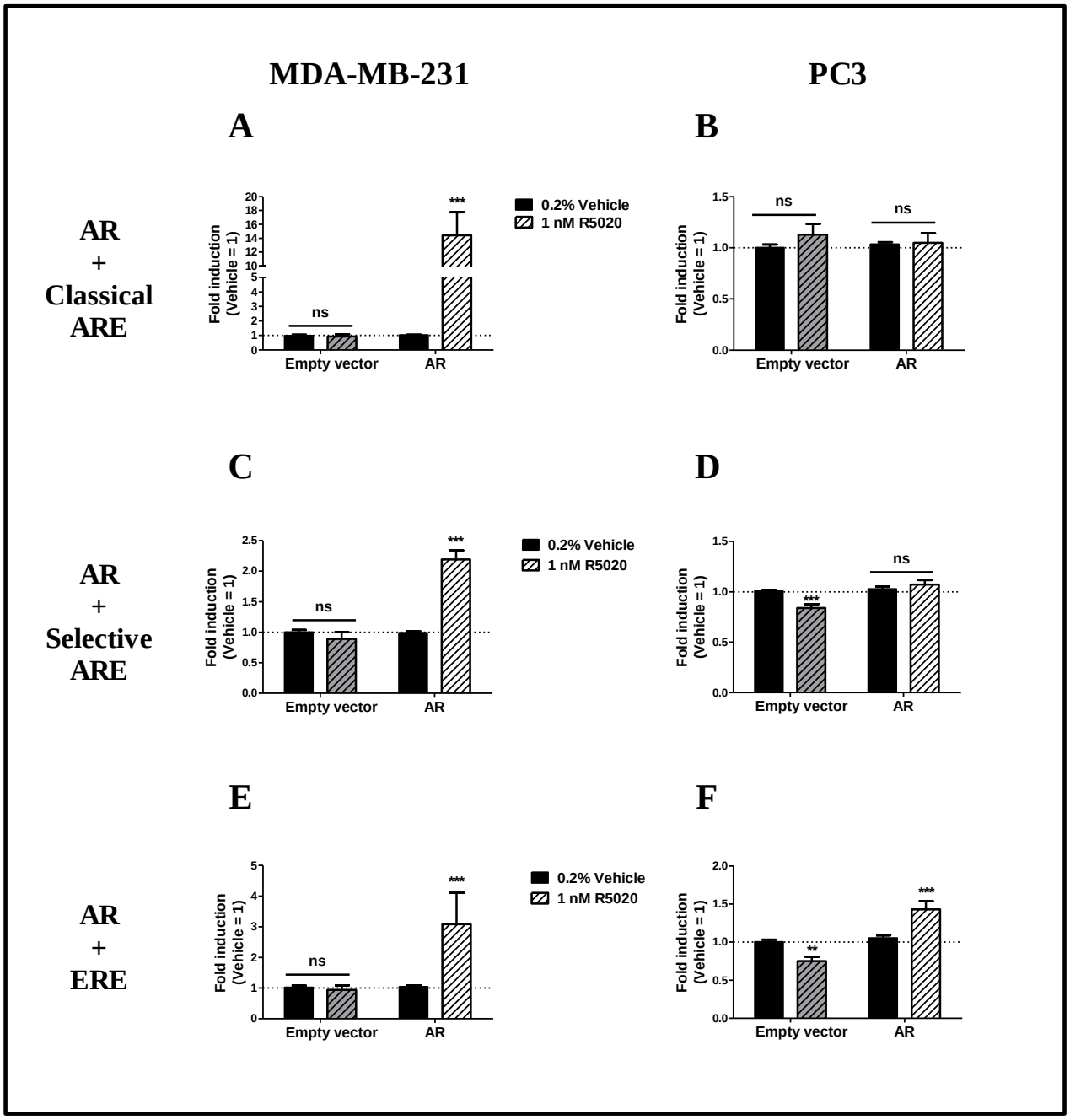


**Figure 3.5: R5020 increased AR-mediated activation at the classical ARE and selective ARE in a cell line-specific manner, while ERE activity was similarly increased in both the MDA-MB-231 and PC3 cell lines.** MDA-MB-231 breast cancer and PC3 prostate cancer cells were transiently transfected with 300 ng of the empty vector (pSG5) (grey bars; A-F) or pSG5-hAR (white bars; A-F) and 3000 ng of the pTAT-PRE-E1b-luc (A-B), 4x*SC*-ARE1.2 (C-D) or the 2xERE-pS2-pGL3 reporter constructs (E-F). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM R5020 for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction is shown with the vehicle control set as 1 and all other responses set relative to this. The results indicate the average of at least two independent experiments, with each condition performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

The results in figure 3.3 indicate that DHT has significant agonist activity via PRB on the PRE, but not the selective ARE or ERE, in both the MDA-MB-231 and PC3 cell lines. Mib on the other hand, activated the PRE and ERE via PRB in only the PC3 cell line (Fig. 3.3 B, F), while significantly increasing the activity of the selective ARE in the MDA-MB-231 cell line (Fig. 3.3 C). Neither Mib nor DHT displayed any agonist activity via PRA on the PRE in the MDA-MB-231 or PC3 cell lines (Fig. 3.4 A-B), while Mib, but not DHT, acts as a PRA agonist on the selective ARE in these cell lines (Fig. 3.4 C-D). Interestingly, DHT showed agonist activity via PRA on the ERE in the MDA-MB-231 cell line (Fig. 3.4 E), but not in the PC3 cell line (Fig. 3.4 F), while Mib had no agonist activity in either cell line (Fig. 3.4 E-F). The results in figure 3.5 indicate that R5020 is an AR agonist on the classical ARE, selective ARE and ERE in the MDA-MB-231 cell line (Fig. 3.5 A, C, E), while it displayed significant AR agonist activity on only the ERE in the PC3 cell line (Fig. 3.5 F). No activation was observed on the response elements in the presence of Mib and DHT (Fig. 3.2 C-H), or R5020 (Fig. 3.5), in the MDA-MB-231 and PC3 cell lines transfected with an empty vector.

Mib and R5020 were initially included in this study due to their reported selectivities for the AR and PR, respectively. As we showed that this is in fact not the case in our cell lines, Mib and R5020 were excluded from future experiments and further investigations were limited to the effects of the natural AR and PR ligands, DHT and $P_4$, as well as the androgenic progestin MPA.

## 3.4 Co-expression of unliganded PRB or PRA with the AR differentially modulated response element activity in a cell line- and/or promoter-specific manner

Given that the AR can bind to an ERE in the promoter of the *PR* gene (Peters et al., 2009), and that blockade of AR activity abrogates PR expression in a preclinical breast cancer model (Wellberg et al., 2017), it is likely that the AR regulates PR expression. Indeed, it is known that the AR and PR are co-expressed in a subset of breast cancers (Ogawa et al., 2008; Niemeier et al., 2010; Park et al., 2010). Considering that crosstalk between co-expressed steroid receptors plays a critical role in breast cancer, we next wanted to investigate the effects of AR and PR co-expression on the activity of the selected response elements in breast cancer cells. Since it is known that PRB and PRA can differentially regulate gene expression in breast cancer, we investigated the effects of both PR isoforms. As the PR is also expressed in prostate cancer tumours (Bonkhoff et al., 2001; Grindstad et al., 2015), we followed a similar investigation in prostate cancer cells.

The MDA-MB-231 and PC3 cell lines were thus transiently transfected with 300 ng of the AR and 3000 ng of either the classical ARE-luc, selective ARE-luc or ERE-luc, in the absence and presence of equimolar (1x) or excess (5x) concentrations of PRB or PRA (indicated throughout by pink bars) (Fig. 3.6 – 3.7). Changes in activity in the presence of both the AR and the unliganded PR isoforms were measured relative to the AR with 1 nM DHT set as 100%. The results show that co-expression of equimolar and excess unliganded PRB with the AR significantly increased activity on the classical ARE in the MDA-MB-231 cell line (Fig. 3.6 A), while unliganded PRA decreased classical ARE activity (Fig. 3.7 A). Similar results were observed in the PC3 cell line (Fig. 3.6 B, 3.7 B). In contrast, selective ARE activity was not modulated by the presence of either concentration of PRB in MDA-MB-231 cells (Fig. 3.6 C), while the activity was increased with an equimolar concentration of PRA (Fig. 3.7 C). In the PC3 cell line, excess PRB increased selective ARE activity (Fig. 3.6 D), while neither concentration of PRA had an effect (Fig. 3.7 D). Interestingly, ERE activity was increased in the presence of equimolar and excess PRB in both the MDA-MB-231 (Fig. 3.6 E) and PC3 (Fig. 3.6 F) cell lines, while for PRA similar results were obtained only at the highest concentration (Fig. 3.7 E-F). Taken together, the data suggests that co-expression of the AR and unliganded PRB or PRA can differentially modulate activity on the response element(s) when compared to AR alone in both the MDA-MB-231 and PC3 cell lines.

In an attempt to explain the observed changes in response element activity when the AR and either PRB (Fig. 3.6) or PRA (Fig. 3.7) were co-expressed, we next investigated AR expression levels in the absence and presence of either PR isoform (Fig. 3.8). Western blot analysis showed that transient transfection of excess PRB or PRA resulted in appropriate increases (~5-fold) in PR protein expression in both the MDA-MB-231 and PC3 cell lines (Fig. 3.8 A-C). Moreover, the results in figure 3.8 D-F show that co-expression of equimolar AR and PRB or PRA did not affect AR expression in either cell line, while excess PRB and PRA decreased AR expression in both cell lines.

## 3.5 Transcriptional activation in cells co-expressing the AR with liganded PRB is dependent on the specific PR ligand, response element and cell line used

Since we have shown that co-expression of the AR with unliganded PRB modulated response element activity in a cell line- and promoter-specific manner, we next investigated how this activity would be influenced when the AR is activated by DHT, and PRB by its natural ligand, $P_4$, or by MPA, the synthetic PR ligand known to also activate the AR. MDA-MB-231 and PC3 cells transiently transfected with the AR and either the classical ARE-luc, selective ARE-luc or ERE-luc in the absence and presence of equimolar (1x) or excess (5x) concentrations of PRB were thus treated with DHT in the absence and presence of $P_4$ or MPA (Fig. 3.9-3.11).

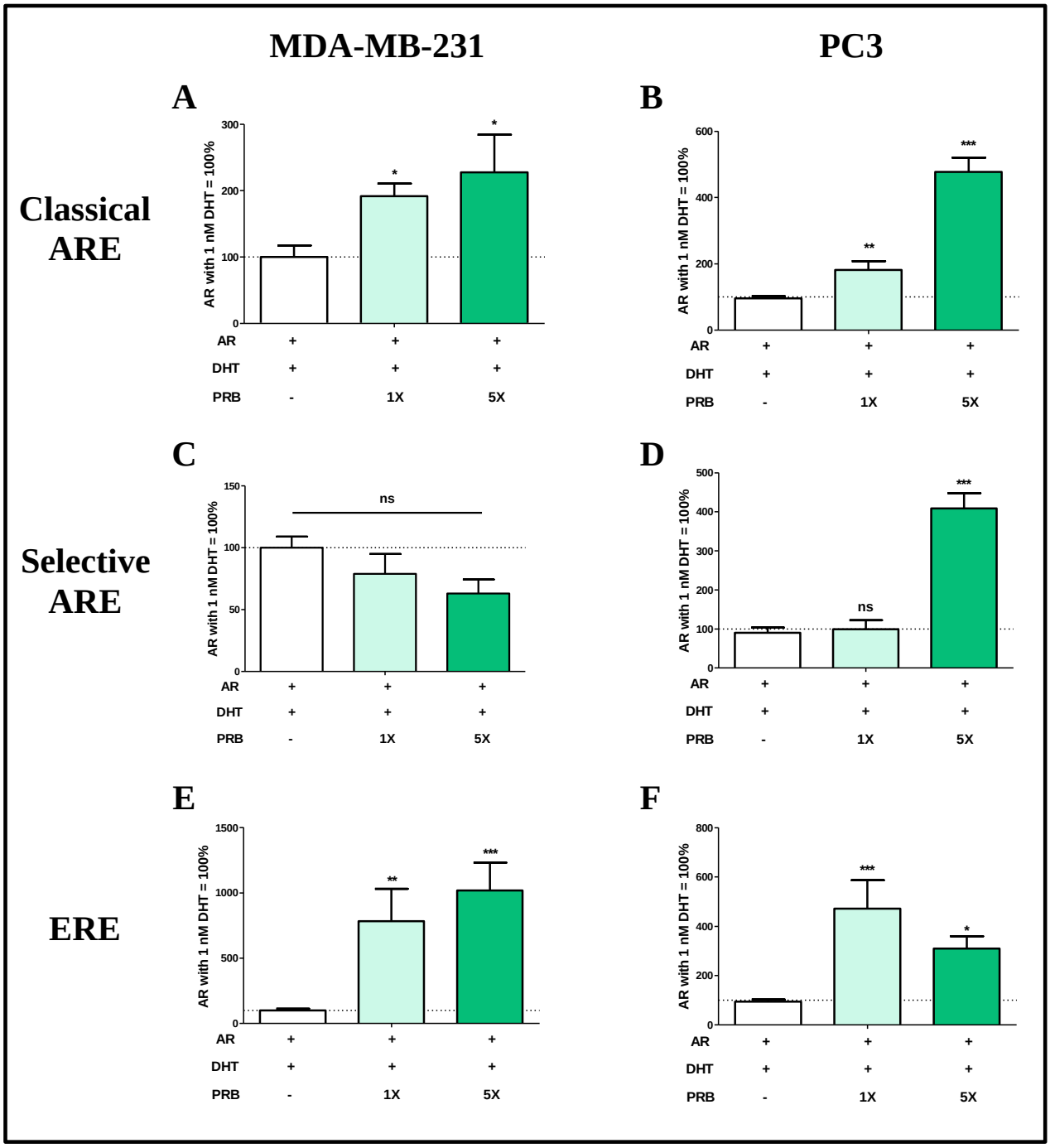


**Figure 3.6: Co-expression of AR and equimolar or excess unliganded PRB increased AR activity on a classical ARE and ERE in the MDA-MB-231 and PC3 cell lines.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-F) together with 3000 ng of either the pTAT-PRE-E1b-luc (A-B), 4x*SC*-ARE1.2 (C-D) or 2xERE-pS2-pGL3 (E-F) reporter constructs in the absence and presence of 300 ng (1x) (blue bars; A-F) or 1500 ng (5x) pSG5-hPR-B (green bars; A-F). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM DHT for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least two independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

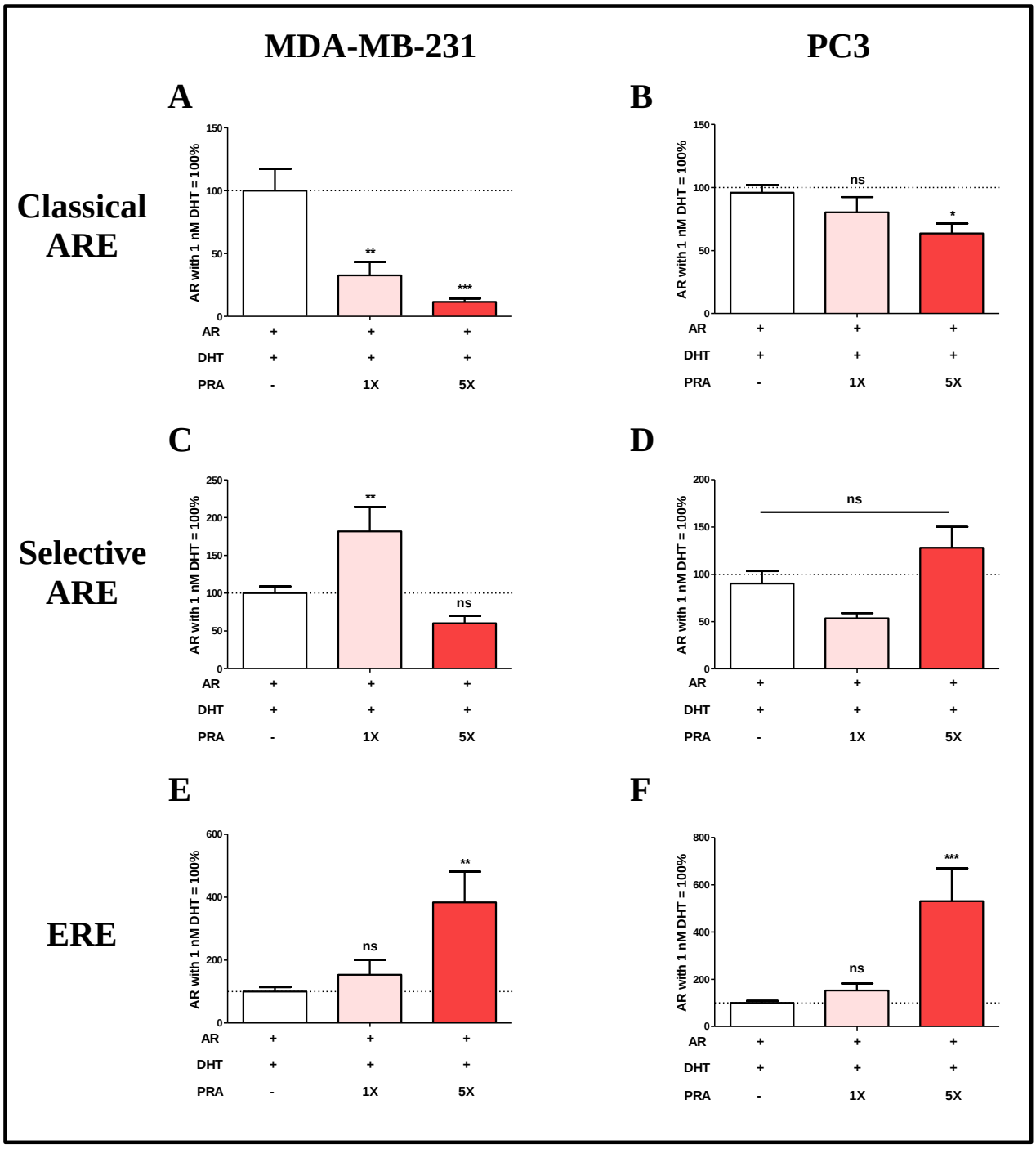


**Figure 3.7: Co-expression of unliganded PRA with AR modulated classical ARE and ERE activity in a concentration-specific manner in both the MDA-MB-231 and PC3 cell line, while selective ARE activity was only influenced in the MDA-MB-231 cell line.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-F) together with 3000 ng of either the pTAT-PRE-E1b-luc (A-B), 4x*SC*-ARE1.2 (C-D) or 2xERE-pS2-pGL3 (E-F) reporter constructs in the absence and presence of 300 ng (1x) (pink bars; A-F) or 1500 ng (5x) pSG5-hPR-A (red bars; A-F). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM DHT for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR was set as 100% and all other responses set relative to this (A-F). The results indicate the average of at least two independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

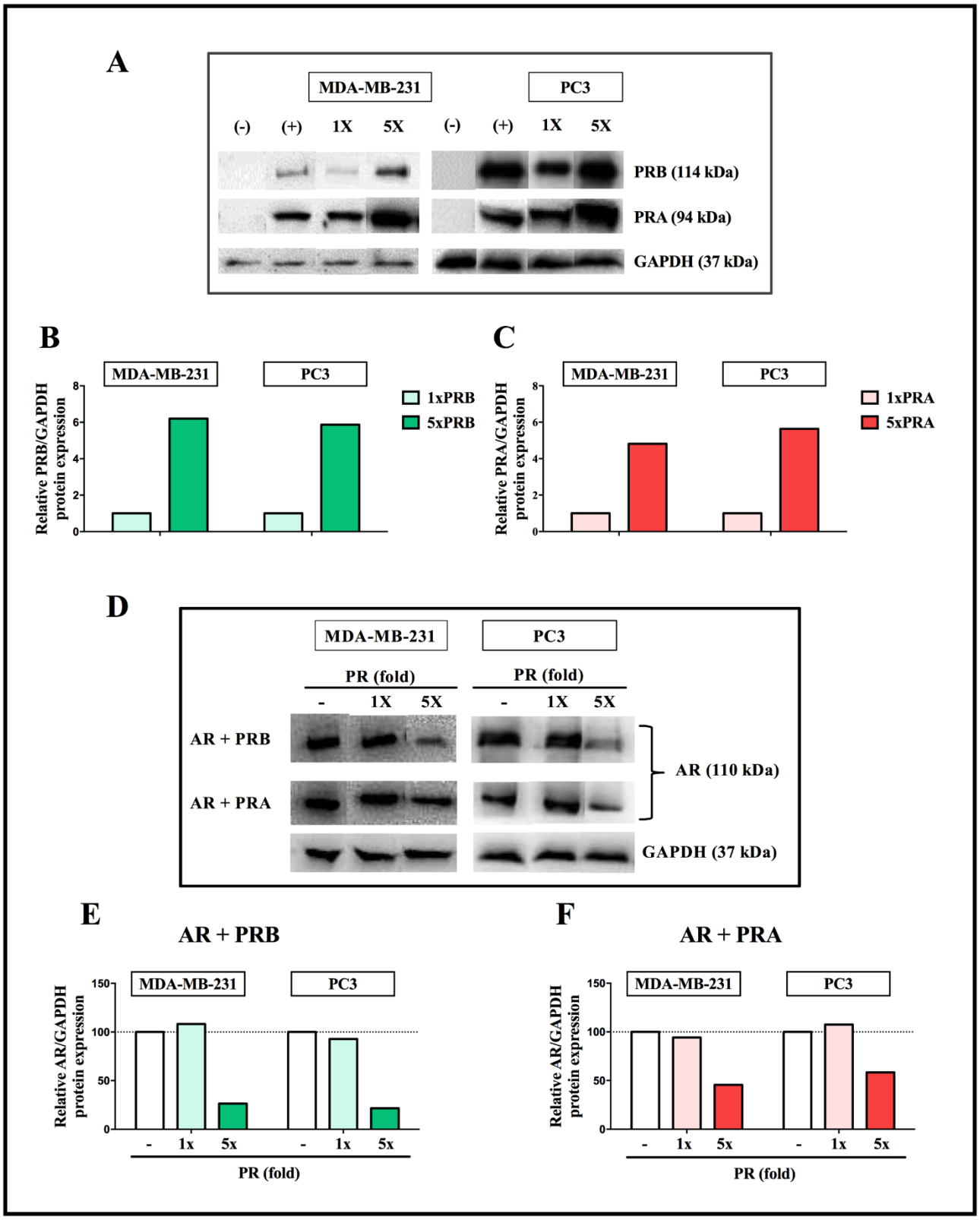


**Figure 3.8 legend on next page.**

**Figure 3.8: Overexpressing excess PR isoforms, but not equimolar concentrations, decreased overexpressed AR protein levels in the MDA-MB-231 breast and PC3 prostate cancer cell lines.** Whole cell extracts were prepared from the MDA-MB-231 breast and PC3 prostate cancer cell lines transfected with either 300 ng (1x) or 1500 ng (5x) pSG5-hPR-B or pSG5-hPR-A in the absence (A) and presence of 300 ng pSG5-hAR (D). Protein lysates were analysed by western blotting, using antibodies specific to PRB, PRA, AR and GAPDH (loading control), respectively (A, D). Western blots were quantified using myImageAnalysis software (B, C, E, F). Protein expression with 1xPRB (blue bars; B) or 1xPRA (pink bars; C) was set as 1 and the expression of 5xPRB (green bars; B) or 5xPRA (red bars; C), respectively, set relative to this. AR expression in the absence of either PR isoform was set as 100% (white bars; E-F) and the expression of the AR in the presence of either PR isoform set relative to this. The results shown are from a single experiment. (-) indicates untransfected MDA-MB-231 and PC3 cells, while (+) indicates PC3 cells transiently transfected with either the PRA or PRB for the western blot of MDA-MB-231 lysates and vice versa.

In the MDA-MB-231 cell line, although both $P_4$ and MPA appeared to augment the increased AR transactivation function by the unliganded PRB on the classical ARE, only the effects of MPA were statistically significant (Fig. 3.9 A, C). Similarly, $P_4$ and MPA significantly increased the activity of unliganded PRB on AR transactivation function in PC3 cells, but only at the 5x concentration (Fig. 3.9 B, D). Interestingly, the addition of equimolar $P_4$ and DHT to MDA-MB-231 cells transfected with only the AR resulted in a significant decrease of DHT activity via the AR, suggesting that $P_4$ is an AR antagonist on this response element (Fig. 3.9 A). Although unliganded PRB did not affect AR activity on the selective ARE in the MDA-MB-231 cell line (Fig. 3.6 C), results in figure 3.10 A show that the activity was significantly increased when PRB was activated by $P_4$, albeit only at the 5xPRB concentration. In contrast, activation by MPA increased AR activity on the selective ARE at both concentrations of PRB (Fig. 3.10 C). In the PC3 cell line, however, the increase of AR transactivation function by excess unliganded PRB was augmented by the addition of $P_4$ and MPA (Fig. 3.10 B, D). Although unliganded PRB did not modulate AR activity on the selective ARE in cells co-expressing equimolar concentrations of the AR and PRB, the activity was significantly increased in the presence of MPA (Fig. 3.10 D). Interestingly, the increased AR activity on the ERE observed in the presence of equimolar concentrations of the AR and unliganded PRB in the MDA-MB-231 cell line, was abrogated when PRB was activated by $P_4$ (Fig. 3.11 A), but not MPA (Fig. 3.11 C). In the PC3 cell line, the increased AR transactivation by only the excess unliganded PRB was amplified by $P_4$ and MPA (Fig. 3.11 B, D). Similar activity was observed for MPA in the MDA-MB-231 cell lines (Fig. 3.11 C).

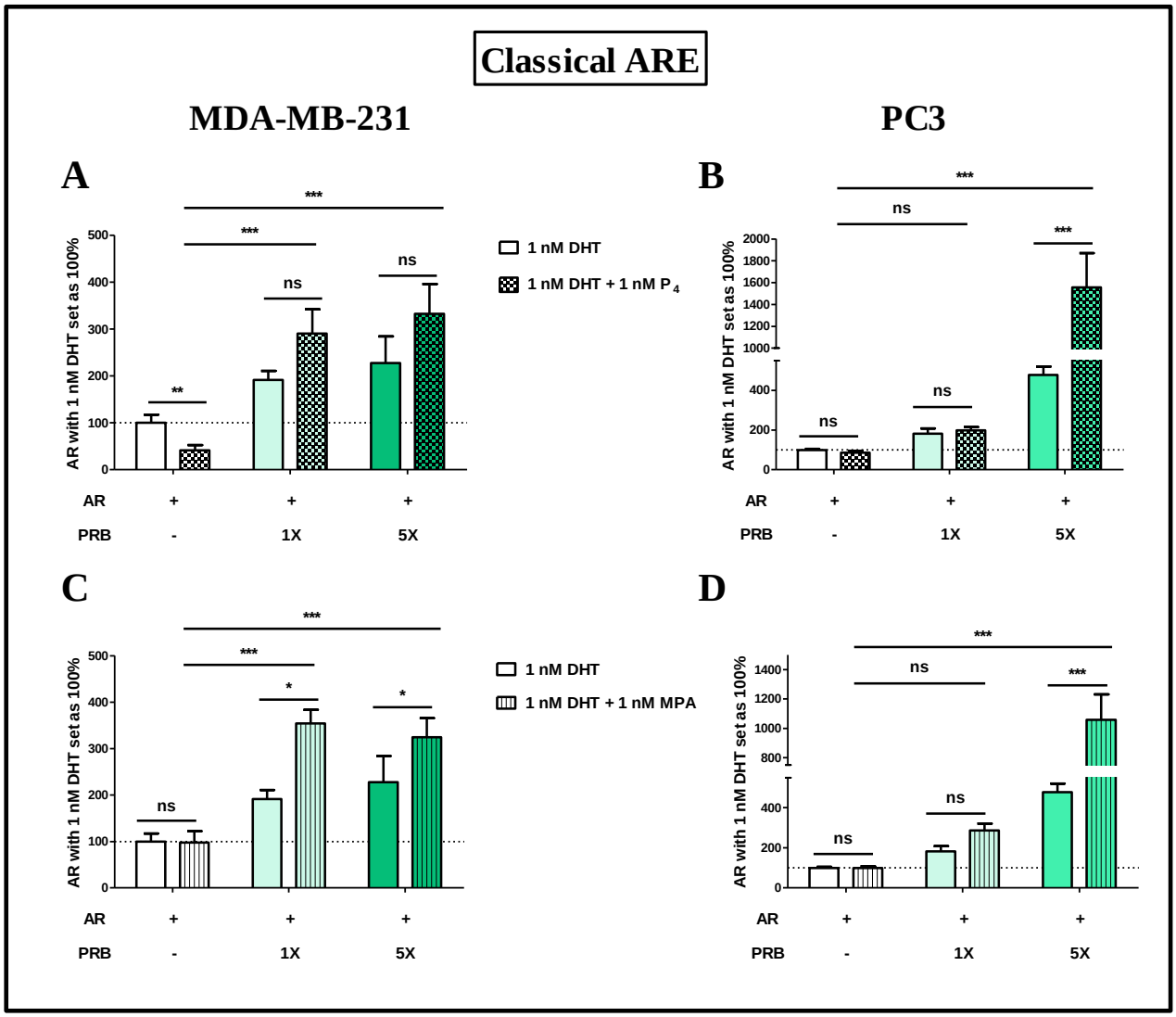


**Figure 3.9: Co-expression of PRB with AR affects classical ARE induction in a ligand- and concentration-specific manner in the MDA-MB-231 and PC3 cell lines, respectively.** The MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the pTAT-PRE-E1b-luc reporter construct in the absence and presence of 300 ng (1x) (blue bars; A-D) or 1500 ng (5x) pSG5-hPR-B (green bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least two independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

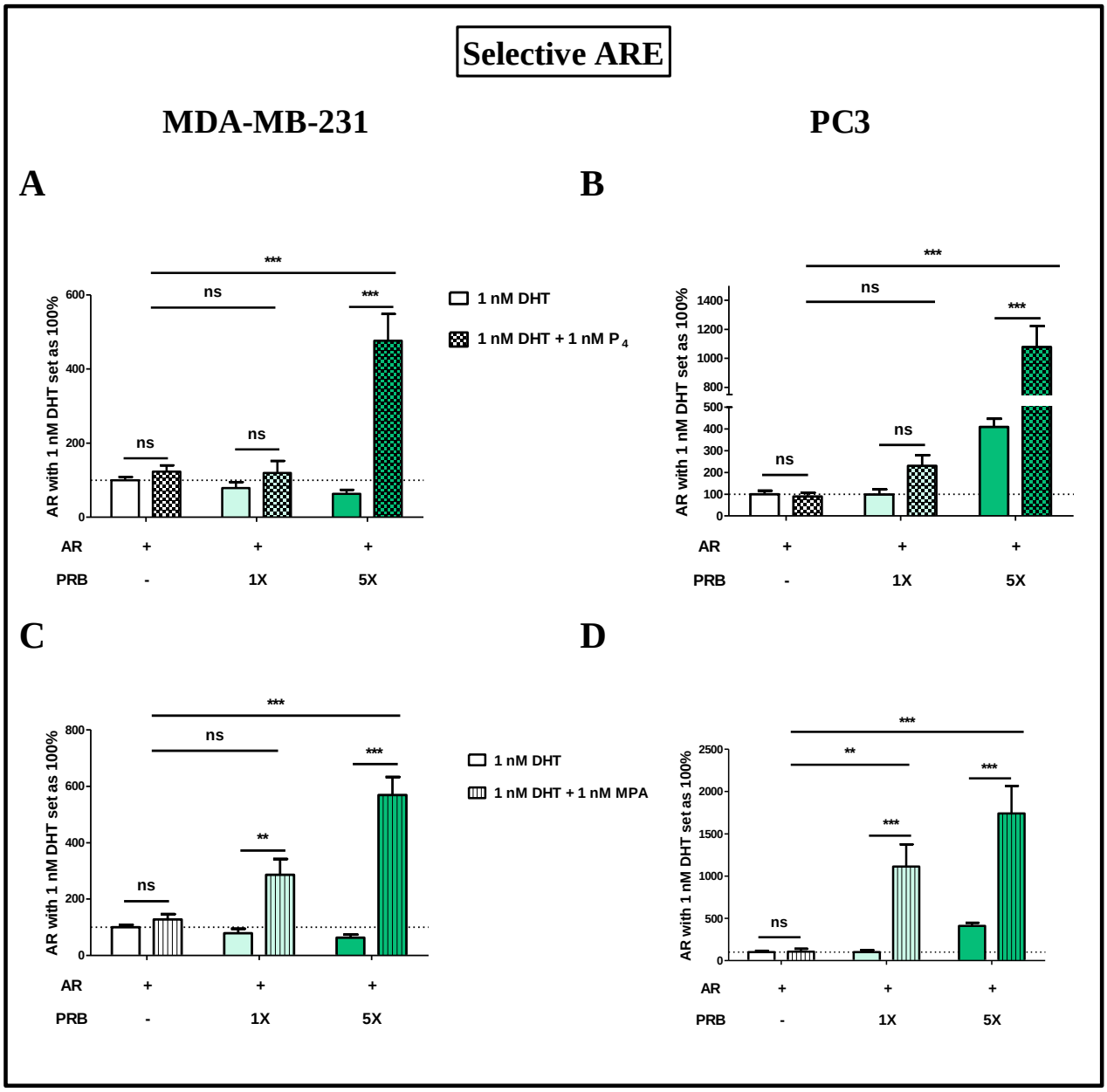


**Figure 3.10: Co-expression of liganded PRB with AR increased selective ARE activation in a concentration-dependent manner in the MDA-MB-231 and PC3 cell lines**. The MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the 4x*SC*-ARE1.2 reporter construct in the absence and presence of 300 ng (1x) (blue bars; A-D) or 1500 ng (5x) pSG5-hPR-B (green bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least two independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

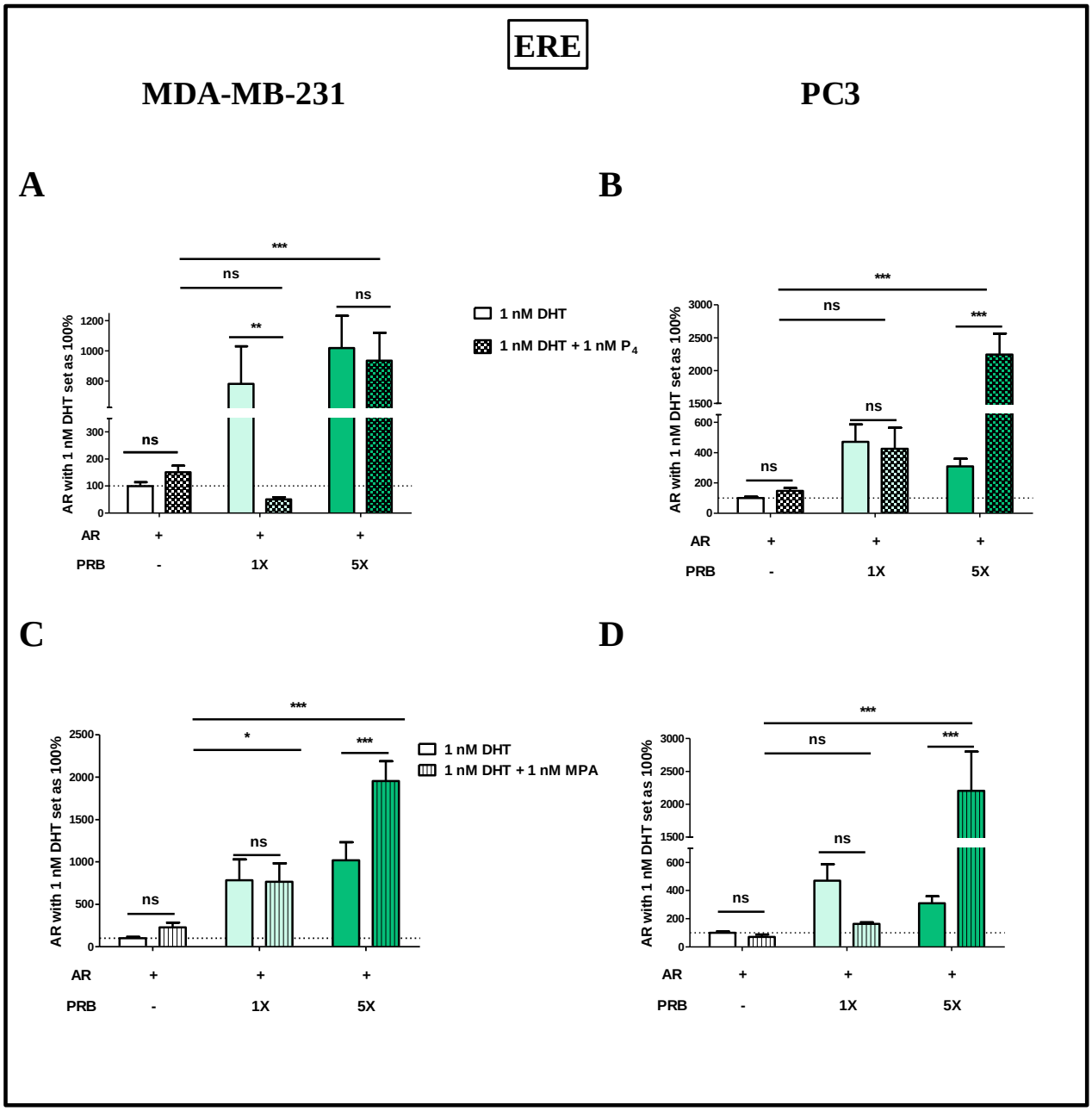


**Figure 3.11: Co-expression of liganded PRB with AR increased ERE activation in a ligand- and concentration-specific manner in the MDA-MB-231 and PC3 cell lines, respectively.** The MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the 2xERE-pS2-pGL3 reporter construct in the absence and presence of 300 ng (1x) (blue bars; A-D) or 1500 ng (5x) pSG5-hPR-B (green bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least two independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

When comparing AR activity on all response elements in the MDA-MB-231 and PC3 cell lines co-expressing the AR and excess liganded PRB, to cells expressing only the AR, it is evident that the activity is increased (Fig. 3.9-3.11). For cells co-expressing equimolar AR and PRB, the activity of the AR on the ERE and selective ARE were increased by MPA, but not $P_4$, in the MDA-MB-231 (Fig. 3.11 A, C) and PC3 cells (Fig. 3.10 B, D), respectively. On the other hand, AR activity on the classical ARE was increased in response to both $P_4$ and MPA at only the excess concentration of PRB in the PC3 cell line (Fig. 3.9 B, D), while both PRB concentrations increased AR activity in the MDA-MB-231 cell line (Fig. 3.9 A, C). Taken together, transcriptional activity in the presence of both AR and PRB and their ligands is promoter-, ligand- and cell line-specific.

### 3.6 Liganded PRA did not affect AR-mediated activity on the classical ARE, but modulated selective ARE and ERE activity in a cell line-, ligand- or concentration-specific manner

Next, we investigated whether liganded PRA would elicit similar effects as PRB when co-expressed with the AR. MDA-MB-231 and PC3 cell lines were transfected with the AR and either the classical ARE-luc, selective ARE-luc or ERE-luc in the absence and presence of equimolar (1x) or excess (5x) concentrations of PRA, and the cells treated as in Section 3.5.

The results show that inhibition of AR transactivation function by unliganded PRA on the classical ARE was not modulated by the presence of the PR ligands in both the MDA-MB-231 and PC3 cell lines (Fig. 3.12). At equimolar concentrations of AR and PRA in the MDA-MB-231 cells, the effect of unliganded PRA on AR activity on the selective ARE was increased by $P_4$, but not MPA (Fig. 3.13 A, C). In contrast, MPA, but not $P_4$, augmented the increase in AR transactivation function by excess PRA on the ERE (Fig. 3.14 A, C). In the PC3 cell line expressing excess PRA relative to AR, both $P_4$ and MPA increased the transcriptional activity of the AR on the selective ARE (Fig. 3.13 B, D), while abrogating the effect of unliganded PRA on the ERE (Fig. 3.14 B, D).

When comparing AR activity in the MDA-MB-231 and PC3 cell lines co-expressing the AR and equimolar PRA, to cells expressing only the AR, transactivation is increased on the selective ARE in only the MDA-MB-231 cell line when $P_4$ and MPA are added (Fig. 3.13 A, C). Similarly, in the presence of the AR and excess PRA, both $P_4$ and MPA increased AR activity on the ERE in the MDA-MB-231 cell line (Fig. 3.14 A, C), and on the selective ARE in the PC3 cell line (Fig. 3.13 B, D). In summary, although the liganded PRA does not influence the effects of DHT via the AR on the classical ARE, effects on the selective ARE and ERE are modulated in a cell line-, promoter or ligand-dependent manner.

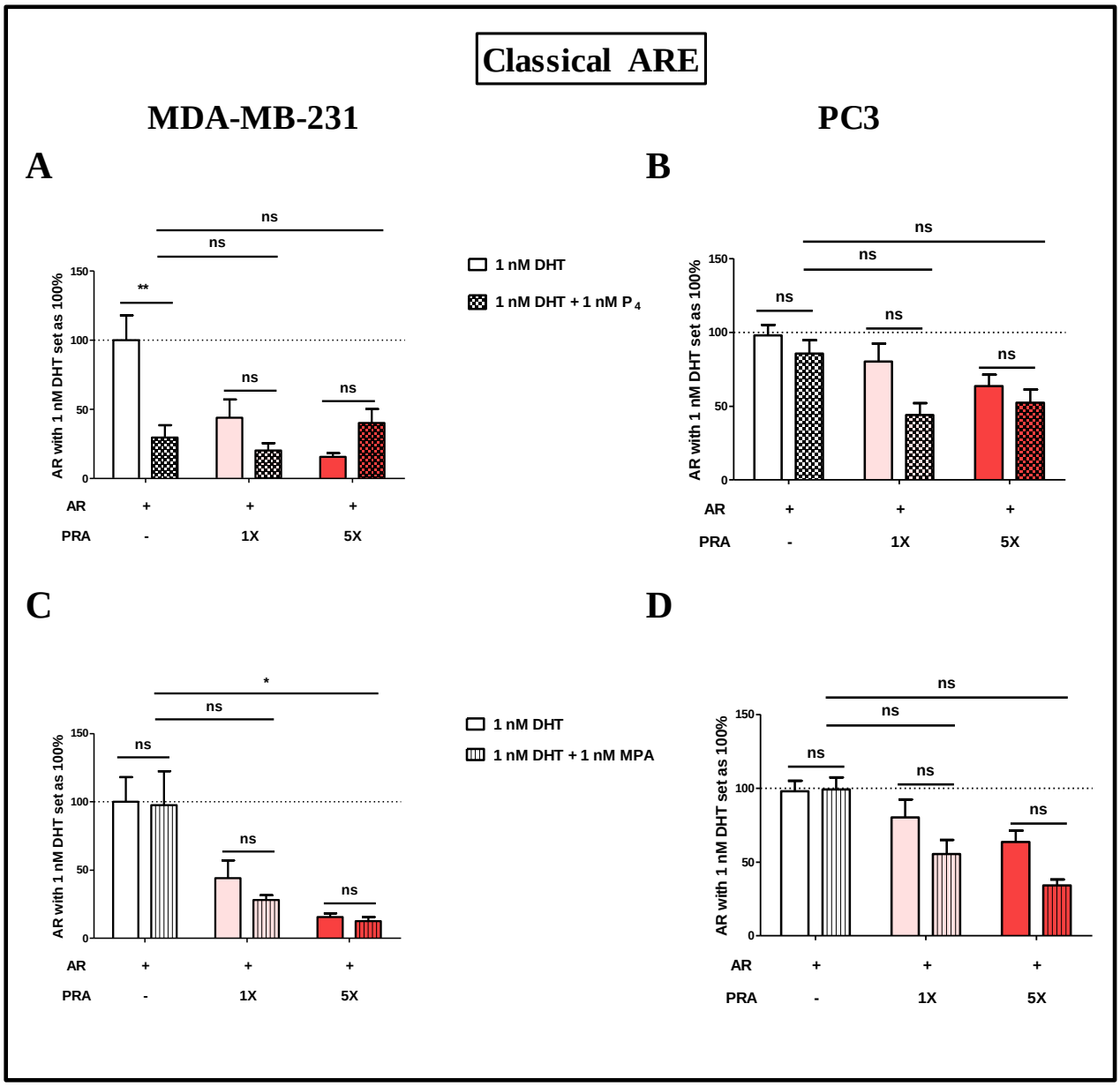


**Figure 3.12: Liganded PRA did not affect the inhibition of AR activity by unliganded PRA.** The MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the pTAT-PRE-E1b-luc reporter construct in the absence and presence of 300 ng (1x) (pink bars; A-D) or 1500 ng (5x) pSG5-hPR-A (red bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least three independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

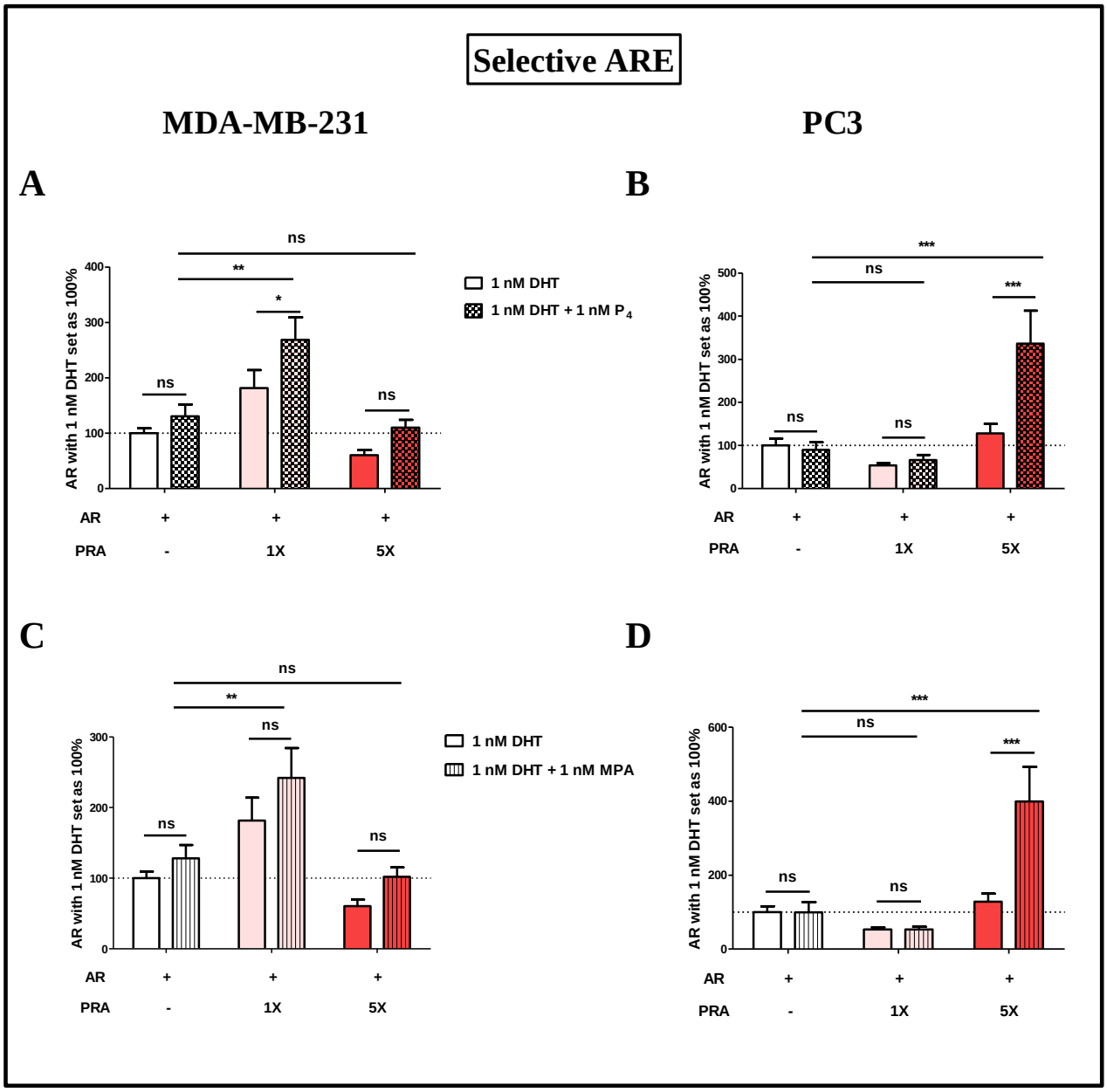


**Figure 3.13: The influence of the liganded PRA on AR activity via the selective ARE is dependent on PR concentration and the cell line used.** The MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the 4x*SC*-ARE1.2 reporter construct in the absence and presence of 300 ng (1x) (pink bars; A-D) or 1500 ng (5x) pSG5-hPR-A (red bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least three independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

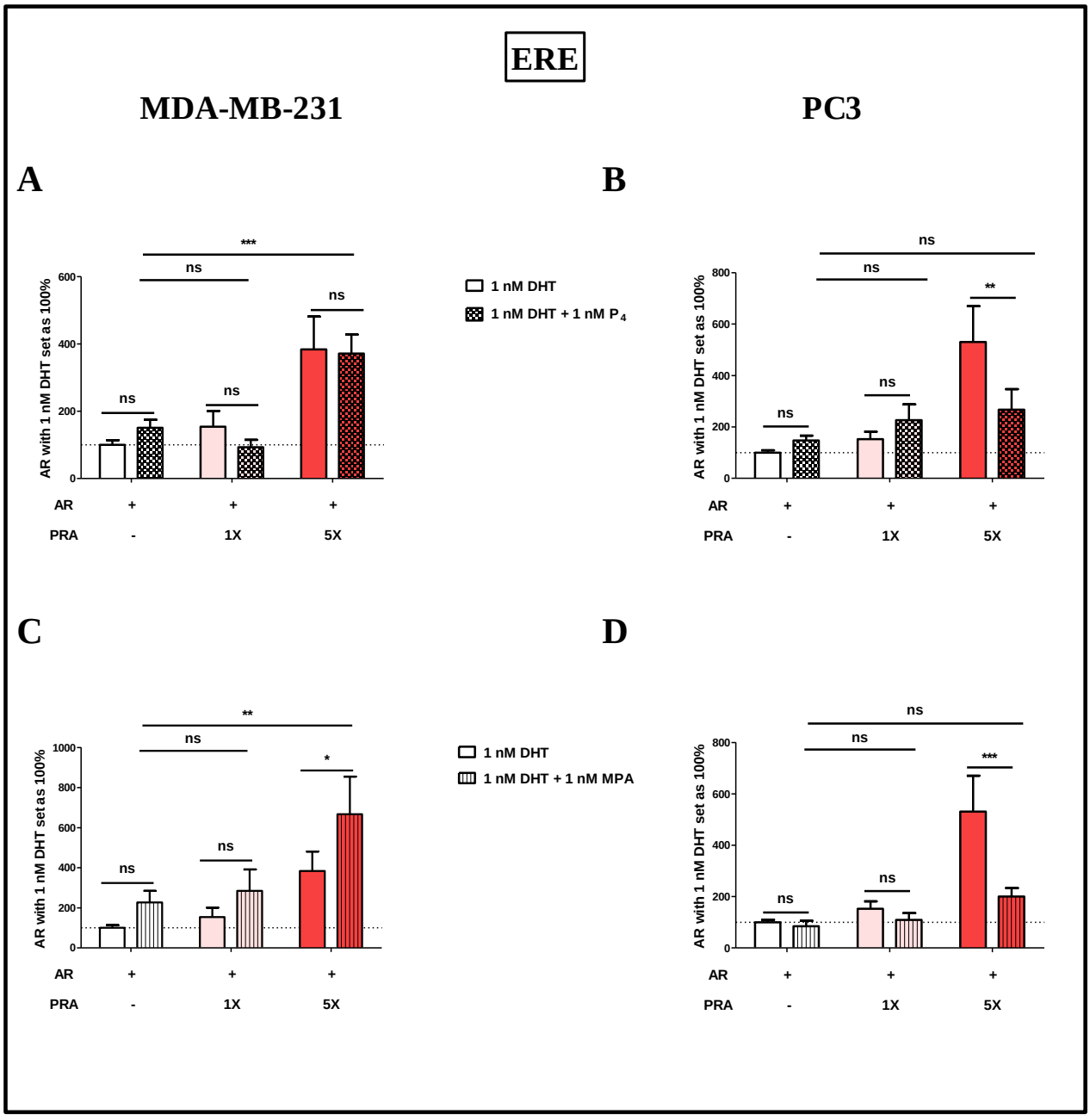


**Figure 3.14: Liganded excess PRA modulated AR activity on the ERE in a ligand- and cell line-specific manner.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the pSG5-hAR (white bars; A-D) together with 3000 ng of the 2xERE-pS2-pGL3 reporter construct in the absence and presence of 300 ng (1x) (pink bars; A-D) or 1500 ng (5x) pSG5-hPR-A (red bars; A-D). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand(s) for 24 hours. Luciferase activity was measured and normalized to total protein concentration. Fold induction at 1 nM DHT via AR only was set as 100% and all other responses set relative to this. The results indicate the average of at least three independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

### 3.7 PRB can transactivate via the selective ARE and ERE, while PRA activates the classical and selective AREs and the ERE in a ligand-specific manner

We have shown that liganded equimolar (1x) and excess (5x) PR isoforms differentially modulate AR activity on all the response elements used in this study (Fig. 3.9-14), and that both the AR and PR isoforms can activate these response elements in the presence of androgens (Fig. 3.2-3.4). Thus, we next wanted to determine the extent to which PR ligands would activate these response elements via the PR isoforms. MDA-MB-231 and PC3 cell lines transiently transfected with either 300 ng (1x) or 1500 ng (5x) PRB (Fig. 3.15) or PRA (Fig. 3.16) and either the selective ARE-luc, ERE-luc or PRE-luc, were thus treated with 1 nM of R5020, $P_4$ or MPA. Cells were also transfected with the empty vector as a control to determine whether transactivation was indeed via the overexpressed PR isoforms.

In both cell lines, only R5020 displayed significant agonist activity via 1xPRB on the selective ARE, while all ligands increased activation of excess PRB on this response element (Fig. 3.15 A-B), suggesting that the selective ARE is not AR-specific as previously reported (Verrijdt et al., 1999). PRB-mediated activity on the ERE was increased by all PR ligands in both cell lines in the presence of excess PRB (Fig. 3.15 C-D). At the 1xPRB, however, only $P_4$ and MPA significantly activated PRB via ERE in the MDA-MB-231 cell line (Fig. 3.15 C), while only R5020 induced a significant increase in the PC3 cell line (Fig. 3.15 D). As expected, the PR ligands activated PRB via PRE in a concentration-dependent manner in both the MDA-MB-231 and PC3 cell lines (Fig. 3.15 E-F).

None of the PR ligands activated 1xPRA in the MDA-MB-231 and PC3 cell lines (Fig. 3.16). However, in both cell lines transfected with excess PRA, R5020 and MPA, but not $P_4$, induced significant increases in PRA activity via the selective ARE (Fig. 3.16 A-B). Although PRA was not activated by any ligand on the ERE in the PC3 cell line (Fig. 3.16 D), only MPA caused activation in the MDA-MB-231 cell line transfected with excess PRA (Fig. 3.16 C). Surprisingly, the PR ligands activated excess, but not equimolar, PRA on the PRE in both the MDA-MB-231 and PC3 cell lines (Fig. 3.16 E-F).

In summary, the results indicate that the progestogens differentially induced PR isoform transactivation via the ERE and selective ARE in the MDA-MB-231 and PC3 cell lines in a PR concentration-dependent manner.

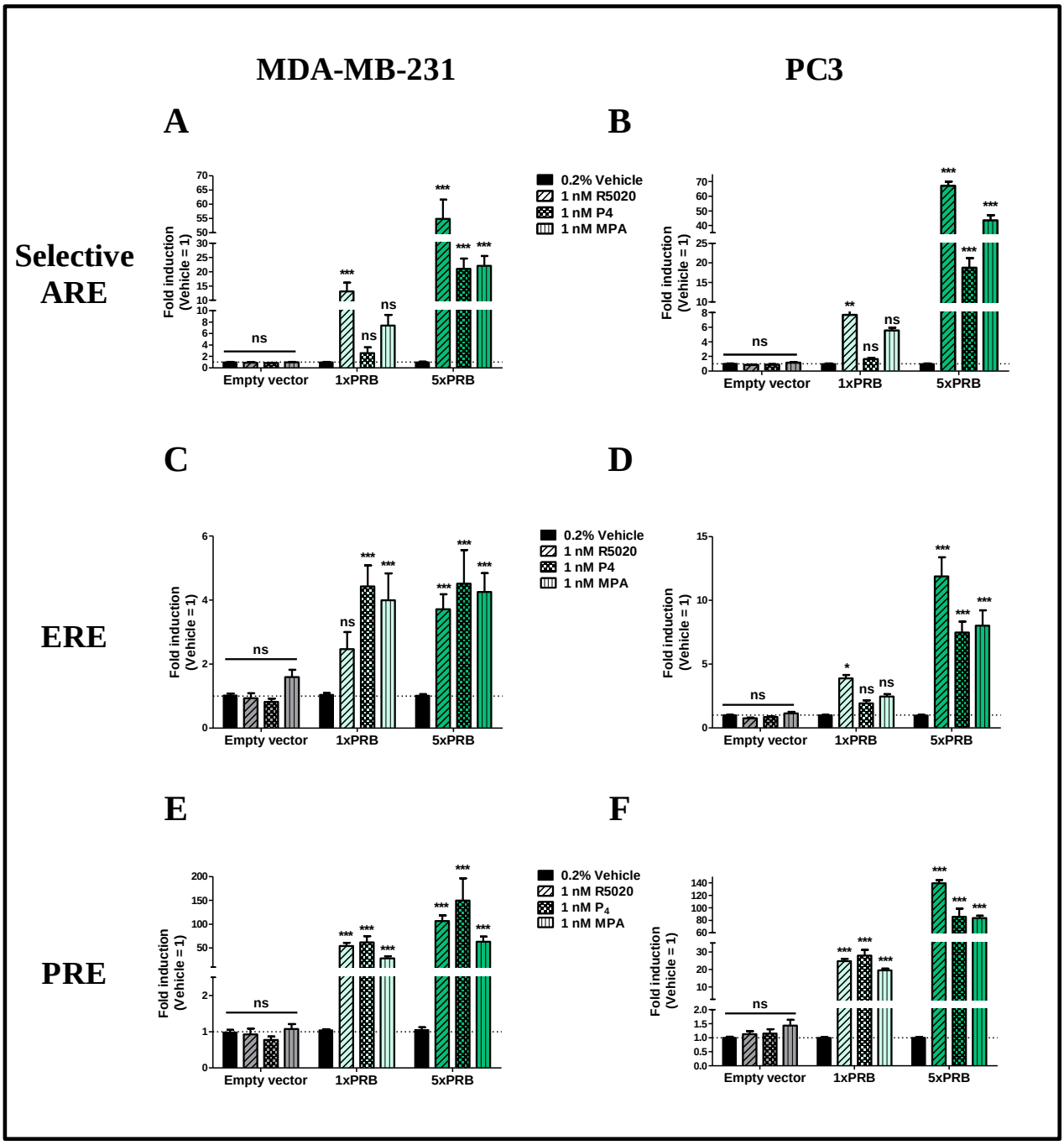


**Figure 3.15: PR ligands differentially induced PRB-mediated transactivation via the selective ARE and ERE in the MDA-MB-231 and PC3 cell lines.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the empty vector (pSG5) (grey bars; A-F), or 300 ng (1x) (blue bars; A-F) or 1500 ng (5x) pSG5-hPR-B (green bars; A-F), together with 3000 ng of either the 4x*SC*-ARE1.2 (A-B), 2xERE-pS2-pGL3 (C-D) or pTAT-PRE-E1b-luc (E-F) reporter constructs. The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand for 24 hours. Luciferase activity was measured and normalized to total protein concentration. The fold induction is shown with the vehicle control of each transfection condition set as 1 and all other responses set relative to this. The results indicate the average of at least four independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (compares all pairs of columns) post -test.

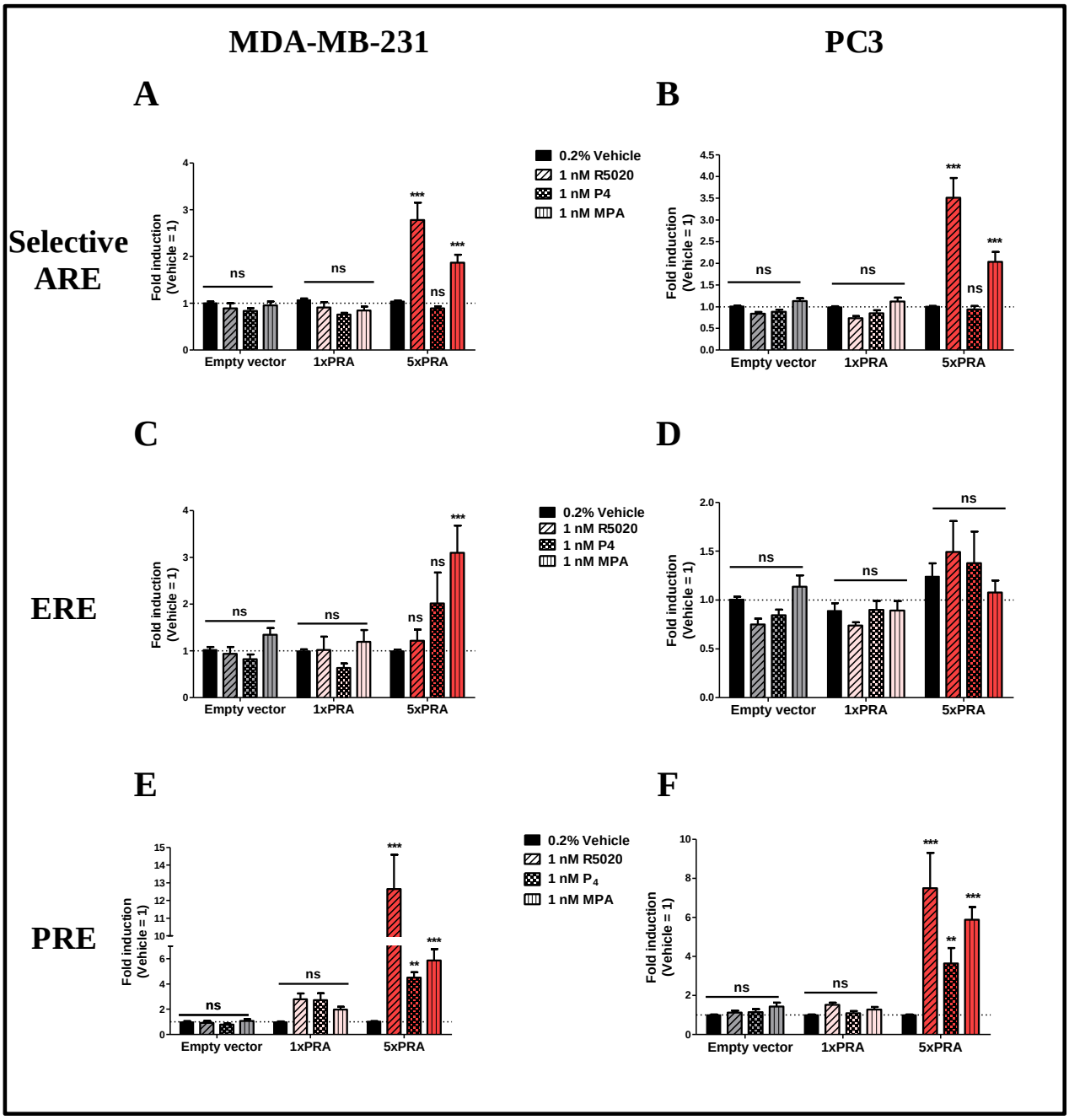


**Figure 3.16: Progestogens differentially induce transactivation on the selective ARE and ERE only in the presence of excess PRA.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng of the empty vector (pSG5) (grey bars; A-F), or 300 ng (1x) (pink bars; A-F) or 1500 ng (5x) pSG5-hPR-A (red bars; A-F), together with 3000 ng of either the 4x*SC*-ARE1.2 (A-B), 2xERE-pS2-pGL3 (C-D) or pTAT-PRE-E1b-luc (E-F) reporter constructs. The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM ligand for 24 hours. Luciferase activity was measured and normalized to total protein concentration. The fold induction is shown with the vehicle control of each transfection condition set as 1 and all other responses set relative to this. The results indicate the average of at least four independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using a column statistics and a one-way ANOVA with a Newman-Keuls (compares all pairs of columns) post -test.

## 3.8 No proliferation was observed in the MDA-MB-231 and PC3 cell lines transiently transfected with the AR

Next, we wanted to investigate whether AR-mediated effects on breast and prostate cancer cell proliferation were influenced by either PRA or PRB. First, we used both the MTT (Fig. 3.17 A-B) and Alamar blue (Fig. 3.17 C-D) cell proliferation assays to determine the effects of DHT via the AR on proliferation of the MDA-MB-231 and PC3 cell lines. These cell lines were thus transiently transfected with either the empty vector or the expression vector for the AR and treated with 0.2% vehicle or 1 nM DHT. As expected, results indicated no proliferation of the MDA-MB-231 and PC3 cell lines in the absence of transfected steroid receptor (empty vector). However, proliferation was also not observed in these cell lines when transiently transfected with the AR. Thus, we were unable to investigate the effects of AR-mediated proliferation of the MDA-MB-231 and PC3 cell lines overexpressing the AR, and thus the influence of the PR isoforms.

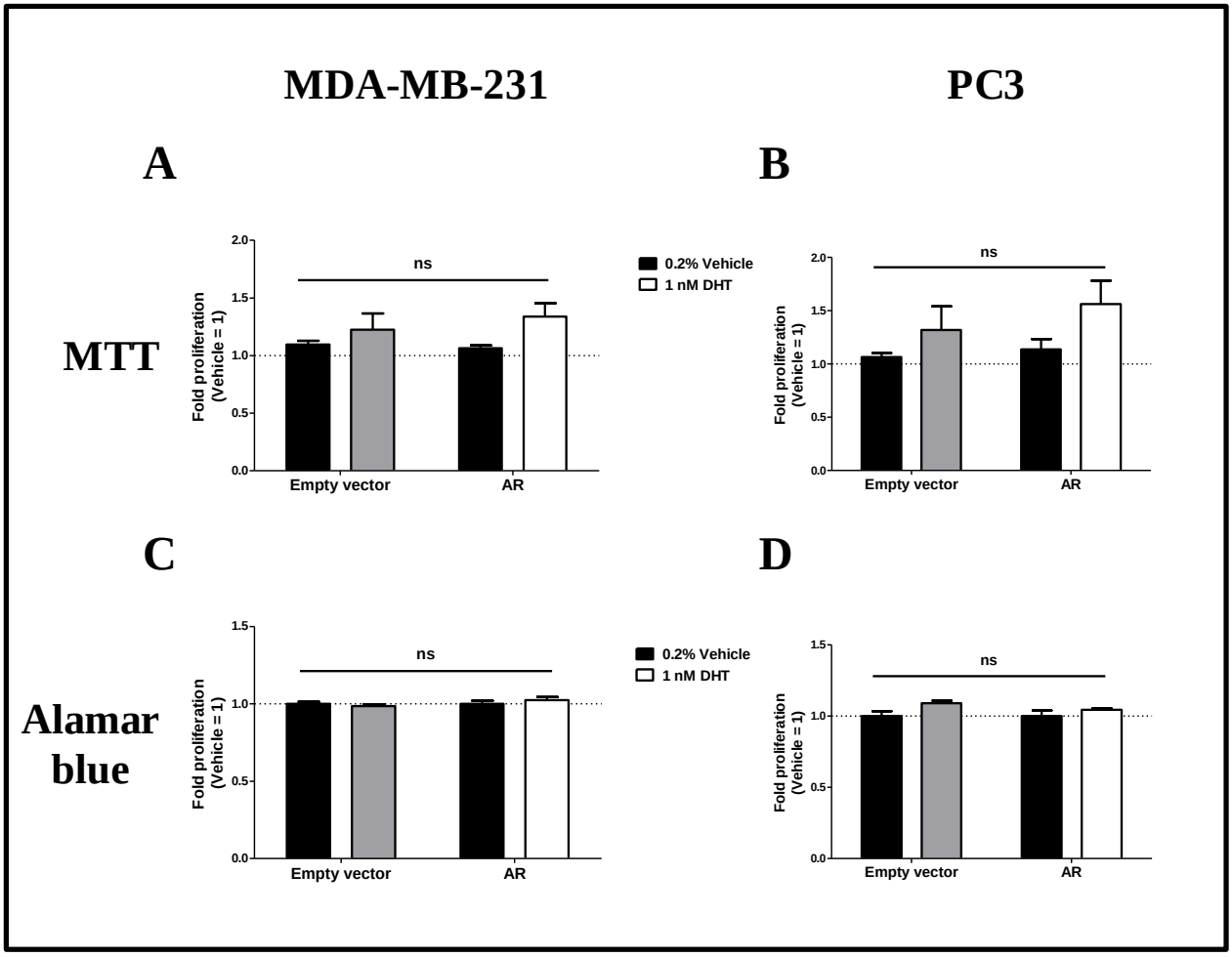


**Figure 3.17: No proliferation was observed in the MDA-MB-231 and PC3 cell lines transiently transfected with the AR.** MDA-MB-231 and PC3 cell lines were transiently transfected with 300 ng of either the empty vector (pSG5) (grey bars; A-D) or the expression vector for the human AR (pSG5-hAR) (white bars; A-D). After 24 hours, the cells were treated with 0.2% v/v vehicle control or 1 nM DHT for 72 hours, and then re-treated with new compounds and

incubated for a further 48 or 168 hours for the MTT and Alamar blue assays, respectively. The fold proliferation was determined by setting the proliferation in the presence of the vehicle as 1 and all other responses relative to that. The results indicate the average of one (C-D) or four (A-B) independent experiment(s), each performed in triplicate (±SEM). Statistical analysis was performed using column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

# Chapter 4

# Discussion

## 4.1 Introduction

Steroid receptors play an important role in both breast and prostate cancer development. The role of the ER is well-established in breast cancer, while that of the AR is well-described in prostate cancer (Sommer and Fuqua, 2001; Shafi et al., 2013). However, the AR is also expressed in about 70-90% of breast cancer tumours and is suggested to play an anti-oncogenic role in ER-positive breast cancer (Peters et al., 2009; Park et al., 2010; Rizza et al., 2014). For example, the AR has been shown to decrease ERα-mediated proliferation (Peters et al., 2009) and increase the expression of anti-proliferative ERβ (Rizza et al., 2014). The PR, although previously thought of as merely a marker of ER functionality, has also recently been reported to contribute to breast cancer development (Ravdin et al., 1992; Hopp, 2004; Allred et al., 2012; Onoda et al., 2015). Interestingly, while a number of studies have previously shown that the AR and PR are co-expressed in breast cancer (Isola, 1993; Ogawa et al., 2008; Niemeier et al., 2010; Park et al., 2010), a very recent study by Wellberg et al. (2017) showed that a functional AR is required for PR expression in a post-menopausal model of obesity-induced breast cancer. These results suggest that the AR, like the ER, may be upregulating the expression of the *PR* gene by a mechanism involving binding of the AR to the ERE in the promoter of the gene. Indeed, Peters et al. (2009) have previously shown that the AR can bind to the ERE in the promoter of the *PR* gene. Interestingly, it has recently also been shown that the natural androgen DHT regulates the expression of both PR isoforms, PRA and PRB, via the AR in human endometrial explants (Babayev et al., 2017). Thus, it is likely that the AR will also drive PR isoform expression in prostate cancer, suggesting that the AR and PR may also be co-expressed in prostate cancer. Indeed, it is known that the PR is expressed in prostate cancer tumours and that its expression is increased with tumour progression (Bonkhoff et al., 2001; Grindstad et al., 2015). To the best of our knowledge, there are currently no studies investigating the possible crosstalk between the AR and PR in breast or prostate cancer. As the AR and PR may contribute to the development of breast and prostate cancer by upregulating the expression of genes known to be involved in the development and progression of these cancers, we investigated the transactivation of gene expression in this study. We focussed on the transactivation function of the AR and PR isoforms on different steroid response elements in the MDA-MB-231 breast cancer and PC3 prostate cancer cell lines. Specifically, we used the classical ARE/PRE that both the AR and PR isoforms are known to activate (Beato et al., 1996), the selective ARE which is reported to be AR-specific (Verrijdt et al., 1999), as well as the binding motif for the ER, the ERE, since the AR has previously been shown to bind to this response element (Peters et al., 2009). Most importantly, we investigated whether AR transactivation function on these steroid response elements was modulated by the presence of PRB or PRA.

## 4.2 Validating the model system

First, we confirmed previous reports in the literature indicating that the MDA-MB-231 breast cancer cell line and PC3 prostate cancer cell line do not endogenously express the PR isoforms or the AR (Fig. 3.1) (Lin et al., 2001; Lai et al., 2009; Nowakowska et al., 2016). Reports of GR expression in the MDA-MB-231 cell line (Abduljabbar et al., 2015; Fietz et al., 2017) and ERα expression in the PC3 cell line (Kim et al., 2002) were also confirmed. Although it has previously been shown that ERβ is present in the PC3 cell line (Kim et al., 2002), we did not detect ERβ expression in these cells, likely due to the lack of an optimal ERβ antibody (Fig. 3.1). Nonetheless, the results suggested that the MDA-MB-231 and PC3 cell lines were appropriate model cell lines for comparing the activities of the AR and PR isoforms.

Next, we validated the promoter-reporter assays in the MDA-MB-231 and PC3 cell lines by showing that PRB was transcriptionally active via the PRE in the presence of the PR ligands, R5020, $P_4$ and MPA (Fig. 3.2 A-B) (Vegeto et al., 1993; Hagan et al., 2011; Izzo et al., 2014). Since the PRE also serves as a classical ARE (Africander et al., 2014), we showed that the AR was able to activate this response element, as well as the selective ARE, in the presence of the androgens, Mib and DHT (Fig. 3.2 C-F). Unexpectedly, no activation was observed for the progestin MPA (Fig. 3.2 C-F), which has previously been shown to induce AR activation on both the classical and selective AREs in the COS-1 monkey kidney cell line (Africander et al., 2014). The difference in response between this study and that of Africander and co-workers may be due to differences in the amount of transfected AR and the use of different cell lines. Finally, in agreement with the study by Peters et al. (2009) who showed that DHT-bound AR could bind to the ERE, we showed that DHT activated the transactivation function of the AR via an ERE in both the MDA-MB-231 and PC3 cell lines (Fig. 3.2 G-H). Interestingly, Mib, but not MPA, also induced AR-mediated ERE activation in both cell lines (Fig. 3.2 G-H), while MPA only caused this response in the MDA-MB-231 cell line (Fig 3.2 G). These results suggest that the effects of MPA via the AR on an ERE are cell-specific and may be attributed to the fact that different cell lines may express different co-activators that are needed for transcription (Beato et al., 1996). In addition, since it is known that MPA can be metabolised (Utaaker et al., 1988; Kobayashi et al., 2000; Mimura et al., 2003), cell-specific responses to MPA may also be due to differences in the metabolism of MPA between these cell lines.

Prior to co-expressing the AR with PRA or PRB, we determined whether the AR and PR ligands used in this study were specific to their respective receptors. The initial rationale was to use Mib to activate the AR and determine whether PRA or PRB could influence AR-mediated transactivation, as this ligand is frequently used in the literature as an AR-specific agonist (Miki et al., 1988). However, we

found that Mib exhibited significant progestogenic activity via PRB in a cell line- and promoter-specific manner (Fig. 3.3), while only displaying activity via PRA on the selective ARE (Fig. 3.4). Upon closer inspection of the literature, we found a study from many years ago showing that Mib could bind to the PR in the human prostate with comparable affinity to the potent synthetic PR agonist, R5020 (Murthy et al., 1986). However, binding affinity is a poor indicator of transcriptional activity (Bain et al., 2014), and the study by Murthy and co-workers did not indicate whether Mib has PR agonist or antagonist activity. Although this study also did not specify which PR isoform Mib bound to, another study has shown that Mib decreased MCF-7 breast cancer cell proliferation via both PRB and PRA (Cops et al., 2008). Considering that Mib is widely used as an AR-specific agonist in the steroid receptor field, our results highlight the previously underappreciated fact that Mib may also be a PR agonist. Thus, caution should be exercised when interpreting the effects of Mib on transactivation via specific response elements in a system that expresses both the AR and PR.

Interestingly, the natural ligand for the AR, DHT, showed significant agonist activity via PRB on the classical ARE in both cell lines (Fig. 3.3 A-B), while only activating PRA on the ERE in the MDA-MB-231 cell line (Fig. 3.4 E). These results are consistent with another study in which DHT was found to have agonist activity via PRB, but not PRA, on a PRE/classical ARE in the human T47D breast cancer cell line (Ghatge et al., 2005). Interestingly, increased expression of the *PSA* gene, a prostate cancer marker which contains a classical ARE in its promoter (Lai et al., 2009), has been associated with increased breast cancer tumour size and histological grade (Black et al., 2000). Thus, the fact that DHT was able to induce PRB-mediated classical ARE activation in the PC3 cell line may provide a putative mechanism through which increased PR expression may lead to prostate cancer progression (Bonkhoff et al., 2001; Grindstad et al., 2015).

Surprisingly, we also found that R5020 induced AR-mediated activation via all the response elements in the MDA-MB-231 cell line (Fig. 3.5 A, C, E), but only on the ERE in the PC3 cell line (Fig. 3.5 F). These results indicating that R5020 is not PR-specific is in agreement with a study showing that it binds the GR (Lippman et al., 1976), and suggests that caution should also be exercised when interpreting the effects of R5020 on the transactivation of ERE-containing target genes in a system that expresses both the AR and PR. Due to the lack of receptor specificity of Mib and R5020, we thus excluded both of these ligands from the co-expression studies. Although we showed that DHT was also not AR-specific, we continued using DHT as it is the natural ligand for the AR.

## 4.3 Unliganded PRB and PRA differentially modulated AR transactivation function via the different response elements

Having validated the proposed experimental model systems, we next investigated the effects of PRB and PRA on AR-mediated transactivation in the absence of PR ligands. The results showed that co-expression of equimolar or excess PRB with AR in the absence of PR ligands increased AR activity on the classical ARE and ERE in a PRB concentration-dependent manner in the MDA-MB-231 and PC3 cell lines (Fig. 3.6 A-B, E-F). In contrast, AR activity was increased only on the selective ARE in the PC3 cell line transfected with excess PRB (Fig. 3.6 C-D).

Understanding the role of the AR and PR isoforms when they are co-expressed is not straightforward. As we have shown that DHT is an agonist of both the AR and PRB on the classical ARE in the MDA-MB-231 and PC3 cell lines (Figs. 3.2 C-D; 3.3 A-B), either one or both receptors could be contributing to the observed increase in response. In an attempt to understand this conundrum, the combined data in figures 3.3 A-B and 3.6 A-B were re-plotted to show how the presence of the AR influences the activity of DHT via PRB (Fig. 4.1 A-B). When the data is presented in this manner, the results show that the activity of PRB on the classical ARE is significantly increased in the presence of the AR, and that neither the AR (Fig. 3.2 C-D) nor PRB (Fig. 4.1 A-B) alone could induce the increased relative fold induction observed when these receptors are co-expressed (Fig. 3.6 A-B). Collectively, these results suggest that both the AR and PR are required for the increased response observed on the classical ARE (Fig. 3.6 A-B). Since it is known that the AR and GR can form functional heterodimers (Chen et al., 1997), and considering the structural similarities between the AR and PR isoforms, it is likely that this increase in activity may be due to a direct interaction between the AR and PRB.

When investigating the effects of PRA on AR activity, the results showed that PRA decreased AR-mediated activity on the classical ARE at both concentrations of PRA in the MDA-MB-231 cell line, but only at the excess concentration in the PC3 cell line (Fig. 3.7 A-B). Interestingly, a study by Vegeto et al. (1993) has previously shown that PRA decreases AR-mediated transactivation on the classical ARE/PRE-containing mouse mammary tumour virus (MMTV)-luc promoter transfected into CV-1 monkey kidney cells. In addition, the results from the western blot analysis (Fig. 3.8 D, F) indicated that inhibition of AR activity by excess PRA may be due to PRA decreasing AR protein levels. However, as both the AR and excess PRA can activate the classical ARE (Fig. 3.2 C-D; 3.16 E-F), the decrease in AR activity in the presence of the excess concentration of PRA in both cell lines (Fig. 3.7 A-B) may also be due to these receptors competing for binding to the response element. In contrast to the results on the classical ARE, an increase in AR transactivation

on the selective ARE was observed with only equimolar unliganded PRA in the MDA-MB-231 cell line (Fig. 3.7 C), while neither PR concentration had an effect on AR activity on this response element in the PC3 cell line (Fig. 3.7 D). Although the precise mechanism for the increase at the equimolar, but not excess, PRA concentration (Fig. 3.7 C) is not known, results from the western blot analysis (Fig. 3.8 D, F) showed that the increase in AR activity is not due to increased AR expression, as AR protein expression was not modulated by equimolar PRA (Fig. 3.8 D, F). The fact that excess PRA had no effect on AR transactivation function (Fig. 3.7 C), while decreasing AR expression (Fig. 3.8 D, F) suggests that the relative expression levels of PRA to AR determine AR activity on the selective ARE when these receptors are co-expressed in the MDA-MB-231 cell line.

The fact that DHT displayed agonist activity via both the AR and PRA on the ERE in the MDA-MB-231 cell line (Figs. 3.2 G; 3.4 E), suggests that the increase in ERE activation upon co-expression of excess PRA with AR may also be mediated by either or both receptors (Fig. 3.7 E). To gain an understanding of the role of these receptors, the data in figure 3.7 E was re-plotted to observe how the effects of PRA on the ERE were modulated in the presence of the AR (Fig. 4.1 C). Yet again, the replotted data indicated that both receptors were involved in the increased response observed on the ERE (Fig. 3.7 E). In contrast, since DHT did not induce PRA activity via the ERE in PC3 cells (Fig. 3.4 F), the increased response by DHT on this response element, when the AR and PRA are co-expressed, is due to the PR augmenting the transactivation function of the AR (Fig. 3.2 H; Fig. 3.7 F).

Taken together, the results indicate that both the AR and unliganded PRB or PRA are required for increased transactivation observed on the different response elements in both cell lines, with the exception of PRA on the classical ARE which decreased AR activity. The former results suggest that either PR isoform may augment AR transactivation on target genes containing the selective ARE or ERE, thereby increasing AR-mediated breast and prostate cancer tumorigenesis. However, PRA may also play a protective role when expressed with the AR by limiting AR-mediated effects on oncogenes containing a classical ARE/PRE sequence. Moreover, the fact that both $P_4$ and DHT can increase PR transactivation function via the selective ARE and ERE suggests that the PR isoforms may regulate genes containing these response elements in their promoter regions, such as the *PSA* gene, a prostate cancer marker which, in addition to the classical ARE, also contains a selective ARE, or the ERE-containing *bcl*-2 gene which regulates apoptosis in breast cancer. This may explain a putative mechanism by which PR isoforms mediate oncogenic effects in breast and prostate cancer.

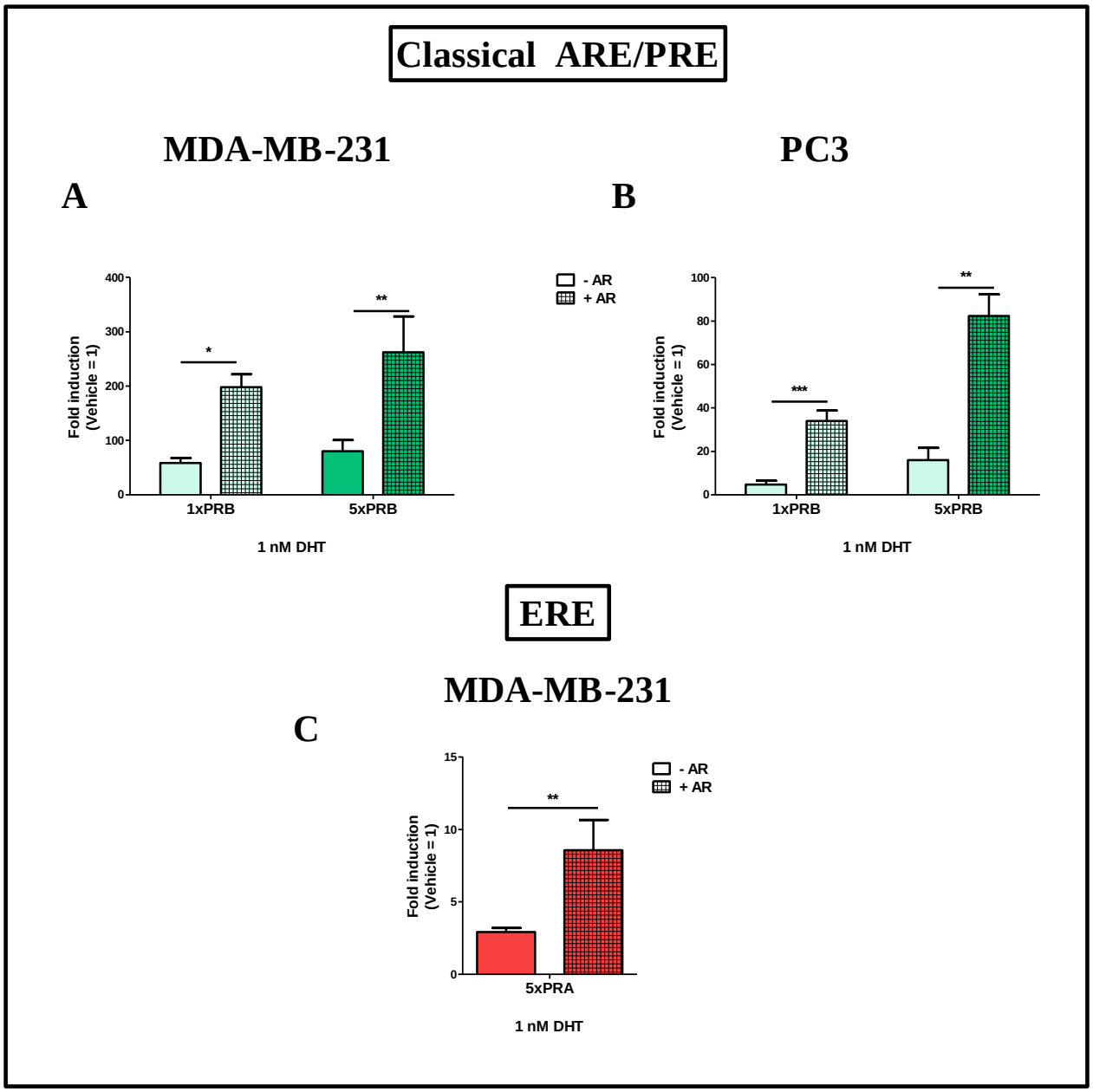


**Figure 4.1: The AR increased equimolar and excess PRB transactivation function on the classical ARE in the MDA-MB-231 and PC3 cell lines, while increasing the activity of excess PRA on the ERE in the MDA-MB-231 cell line.** MDA-MB-231 and PC3 cells were transiently transfected with 300 ng (1x) or 1500 ng (5x) pSG5-hPR-B and 3000 ng of the pTAT-PRE-E1b-luc reporter construct in the absence and presence of 300 ng pSG5-hAR (A-B), or 1500 ng (5x) pSG5-hPR-A and 3000 ng of the 2xERE-pS2-pGL3 reporter construct in the absence and presence of 300 ng pSG5-hAR (C). The next day, the cells were treated with 0.2% v/v vehicle control or 1 nM DHT for 24 hours. Luciferase activity was measured and normalized to total protein concentration. The results are represented as fold induction relative to the vehicle control of each transfection condition. The results indicate the average of at least three independent experiments, each performed in triplicate (±SEM). Statistical analysis was performed using a column statistics and a one-way ANOVA with a Newman-Keuls (comparing all pairs of columns) post-test.

## 4.4 Liganded PRB and PRA differentially modulate AR activity

Our investigations in the presence of the PR ligands, $P_4$ and MPA, showed that the activated PRB increased AR activity on the selected response elements in a ligand-, cell line- and promoter-specific manner (Fig. 3.9-3.11). Although the presence of PR ligands did not modulate the decrease in AR transactivation function on the classical ARE by unliganded PRA (Fig. 3.12), the effects of liganded PRA on the selective ARE and ERE were dependent on the specific PR ligand, the concentration of PRA and the cell line (Fig. 3.13-3.14).

The effects of steroid receptors are complex and dependent on various factors, including receptor density, the type of ligand, the context of the promoter landscape and the cellular milieu (Hager et al., 2009). It is known that different ligands induce different steroid receptor conformations, and that alterations of the LBD ultimately affect the structure of the DBD (Hager et al., 2009; Bain et al., 2014). Indeed, $P_4$ and MPA differ in their structures, and these ligands differentially modulate the conformation of the PR (Duax et al., 1978; Allan et al., 1992). In addition, it is known that the interaction of both $P_4$ and MPA with the PR alters the conformation of the LBD, which influences the structure of the DBD and therefore determines the sequence to which the PR can bind (Duax et al., 1978; Raynaud et al., 1980; Spilman et al., 1986). As it is known that receptor density determines transcriptional activity and the biological character of a steroid hormone (Zhao et al 2003; Robertson et al., 2013), the concentration-dependent effects of the PR isoforms observed in this study could be explained by the fact that more PR protein was present to activate the various response elements (Fig. 3.8 A-C). The cell-specific differences observed in this study may be attributed to the fact that MDA-MB-231 breast cancer and PC3 prostate cancer cell lines each contain a unique milieu of transcriptional regulators (Litvinov et al., 2006; Conzen, 2008; Bhagwat and Vakoc, 2015). In addition, it is known that breast and prostate cancer cell lines differentially metabolise steroids (Raju et al., 1978; Koh et al., 2001), and that $P_4$, MPA and DHT can be metabolised (Utaaker et al., 1988; Kobayashi et al., 2000; Ji et al., 2003; Wiebe, 2006), thus we cannot exclude the possibility that the ligands are metabolised in one cell line and not the other.

The general trend observed that liganded PR isoforms increased AR activity in both the MDA-MB-231 and PC3 cell lines suggests that the transcriptional activation observed in the presence of either the AR or PR isoforms is magnified when these receptors are co-expressed. The exception to this was liganded PRA abrogating the increase on AR activity by unliganded PRA on the ERE in the PC3 cell line (Fig. 3.14 B, D) and liganded equimolar concentrations of PRB abrogating the increase on AR activity by unliganded PRB on the ERE in the MDA-MB-231 cell line (Fig. 3.11 A). Nevertheless, the results indicating increased activity may explain a mechanism through which

increased expression of either PRB or PRA mediates prostate cancer progression (Grindstad et al., 2015). For example, PRB may enhance AR-mediated effects at various oncogenes (Fig. 3.6; 3.9-3.11), or PRB itself could be activated by either $P_4$ or DHT and activate genes encoding a classical ARE/PRE (Fig. 3.2 A-B; 3.3 A-B) in their promoters, such as the *GATA3* gene known to mediate cell migration, or the *PSA* prostate cancer marker (Lai et al., 2009; Yin et al., 2012). The fact that excess liganded PRA abrogated the increased AR activity by unliganded PRA on the ERE in the PC3 cell line (Fig. 3.14 B, D) suggests that unliganded PRA may increase the transcriptional activity of the AR and thus promote prostate cancer, while the liganded PRA would not. In terms of breast cancer, tamoxifen is used to treat breast cancer patients and functions by inhibiting ER-mediated effects on EREs. However, resistance occurs in some patients (Chang, 2012) and both the AR and PRA have been implicated in this resistance mechanism (Hopp, 2004; De Amicis et al., 2010; Rechoum et al., 2014; Ciupek et al., 2015). We have shown that both the AR and PRA can activate the ERE in the MDA-MB-231 cell line (Fig. 3.2 G; 3.16 C), suggesting that these receptors can mimic the role of the ER in breast cancer. Moreover, AR transactivation on the ERE is augmented by PRA in the MDA-MB-231 cell line (Fig. 3.14 A, C) co-expressing the AR and PRA.

Taken together, these results show that crosstalk occurs between the AR and PR isoforms in both prostate and breast cancer, and may provide a mechanism through which these receptors mediate treatment failure in both cancers.

## 4.5 PRB and PRA differentially transactivate via the selective ARE and ERE

While it is known that PRB and PRA can activate similar gene sets, there are some genes that are only regulated by PRA, and others that are predominantly regulated by PRB (Graham and Clarke, 2002; Kariagina et al., 2008). Thus, it is not surprising that we showed that R5020, $P_4$ and MPA, differentially induced PRB (Fig. 3.15) and PRA (Fig. 3.16) transactivation via the selective ARE and ERE in the MDA-MB-231 and PC3 cell lines. To the best of our knowledge, this is the first study to show that PRB and PRA can transactivate via these response elements in both breast and prostate cancer cells. Importantly, the fact that MPA induced both PRB and PRA transactivation on the ERE in the MDA-MB-231 breast cancer cell line (Fig. 3.15 C; 3.16 C) may provide a mechanism for the well-established link between MPA and increased breast cancer risk in women using MPA in hormone therapy (Collaborative Group on Hormonal Factors in Breast Cancer, 1996; Rossouw et al., 2002; Beral and Million Women Study Collaborators, 2003; Chlebowski et al., 2003, 2009, 2013; Fabre et al., 2007; Hunter et al., 2010; Li et al., 2012; Santen, 2014).

Notably, others have previously shown that the GR, activated by the synthetic agonist, dexamethasone (Dex), can mimic the AR in CRPC by activating the transcription of genes containing a selective ARE, thereby maintaining tumorigenesis (Arora et al., 2013). The authors defined genes containing a selective ARE as those which were highly expressed in the presence of DHT relative to those expressed in the presence of Dex. When considering that the selective ARE used in our study was defined as such due to it displaying selectivity for the AR instead of the GR, it is likely that the selective AREs defined in the Arora et al. (2013) study may be similar to the selective ARE employed in this study. Since it is known that a high degree of structural homology exists between the AR, GR and PR (Gao et al., 2005), it was not surprising that the PR isoforms, like the GR, could mediate transactivation on the selective ARE (Fig. 3.3-3.4; 3.15-3.16). These results suggest that the PR mimics AR activity on both the classical and selective AREs and may explain why prostate cancer patients with increased PR expression are more likely to exhibit treatment failure and higher tumour grade (Bonkhoff et al., 2001; Grindstad et al., 2015). In addition, when considering that recent data has shown that serum $P_4$ levels are increased in CRPC patients treated with abiraterone acetate as well as enzalutamide (McKay et al., 2017; Montgomery et al., 2017), this further implies a mechanism by which the activated PR may mediate oncogenic effects in CRPC.

## 4.6 No proliferation of the MDA-MB-231 and PC3 cell lines was observed

It is known that the transactivation of genes aid in the regulation of cell proliferation in cancer (Nicholson et al., 1995). Since we observed changes in AR transactivation in the presence of the PR isoforms, we next wanted to investigate how the PR isoforms would affect AR-mediated effects on cell proliferation. Using both the MTT and Alamar blue cell proliferation assays, we first aimed to investigate the effects of the AR on proliferation of the MDA-MB-231 and PC3 cell lines transiently transfected with the AR. However, no proliferation was observed in the presence of DHT in either cell line (Fig. 3.17) and we could thus not investigate the putative effects of the PR isoforms on AR-mediated proliferation. Results from previous studies investigating the effects of DHT on the proliferation of MDA-MB-231 cells transfected with the AR are contradictory. While one study has shown proliferative effects (Shen et al., 2017), others have indicated that introduction of the AR into the MDA-MB-231 cell line caused decreased cell proliferation (Garay et al., 2012; Narayanan et al., 2014). Similarly, studies investigating changes in PC3 cell proliferation are contradictory, with a study reporting an increase in cell proliferation upon introduction of the AR (Yuan et al., 1993), while others report a decrease or no effect in PC3 cell growth (Lin et al., 1998; Peng et al., 2008; Schweizer et al., 2008; Zeng et al., 2011). These discrepancies in findings may be due to differences in the amount of AR transfected into the cells or different experimental conditions. For example, studies

showing that the AR decreased proliferation in the MDA-MB-231 cell line used cells stably overexpressing the AR and treated with DHT for 72 hours (Garay et al., 2012; Narayanan et al., 2014), while the study showing a decrease in cell proliferation transiently transfected the AR and treated with DHT for 48 hours (Shen et al., 2017). Notably, one of the studies showing decreased proliferation of PC3 cells transiently transfected with the AR transfected more than forty times less AR than used in this study (Zeng et al., 2011), while the other transfected forty times more (Schweizer et al., 2008).

## 4.7 Conclusions and future work

It is widely accepted that androgen signalling via the AR maintains prostate cancer tumorigenesis, however, the role of the AR in breast cancer is less straightforward and seems to depend on the presence of ERα (Proverbs-Singh et al., 2015). However, breast and prostate cancers also express others steroid receptors such as the PR (Brisken, 2013; Grindstad et al., 2015), and emerging evidence suggests considerable interplay between these steroid receptors in breast cancer (Claessens and Tilley, 2014; Sikora, 2016). Although the role of steroid receptors and their interplay is an area of ongoing research, studies in breast cancer have shown both good and bad prognosis associated with receptor crosstalk. For example, the AR has been shown to decrease ERα activity suggesting a protective role for the AR (Peters et al., 2009), while the activated PR is also protective as it redirects ERα chromatin binding such that ERα-mediated proliferative responses are inhibited (Mohammed et al., 2015). In contrast, the AR has been shown to sustain tumorigenesis in a post-menopausal model of $ER^+/PR^+$ breast cancer, possibly by regulating PR expression, as well as through ER-AR crosstalk (Wellberg et al., 2017). Although some studies have suggested a possible interplay between the AR and ERβ (Muthusamy et al., 2011; Grubisha and DeFranco, 2013) in prostate cancer, the role of steroid receptor crosstalk in prostate cancer is not well-described. To the best of our knowledge, this study is the first to show that both PR isoforms can modulate AR transactivation function on the classical and selective AREs, as well as the ERE, in both breast and prostate cancer cell lines. In addition, we provide novel evidence that the PR isoforms, particularly the more transcriptionally active PRB, mimic AR activity on the selected response elements. In terms of prostate cancer, the results presented in Chapter 3 thus provide impetus to clinical studies reporting that high PR expression in prostate cancer is a poor prognostic factor (Bonkhoff et al., 2001; Grindstad et al., 2015). Furthermore, the results showing that the AR and PR isoforms can transactivate via the ERE in the MDA-MB-231 cell line may provide a mechanism through which these receptors can mediate resistance to breast cancer treatments (Hopp, 2004; De Amicis et al., 2010; Rechoum et al., 2014; Ciupek et al., 2015).

Overall, we observed that PRB, whether in the absence or presence of PR ligands, generally upregulated AR transactivation on the various response elements, including the ERE through which ERα mediates oncogenic actions in breast and prostate cancer. These results suggest that PRB may enhance AR-mediated oncogenic effects in prostate and/or breast cancer by increasing the expression of genes containing the selective ARE sequence in prostate cancer, or ERE sequences in both prostate and breast cancer. While AR transactivation function was also increased by PRA on the selective ARE and ERE, PRA decreased AR-mediated transactivation on the classical ARE. Understanding the role of PRA is thus more complex than that of PRB. Although PR expression is increased in prostate cancer, the relative expression levels of the PR isoforms is unknown (Grindstad et al., 2015). In contrast, several studies have shown that the upregulation of PRA relative to PRB in breast cancer is associated with poor prognosis (Hopp, 2004; Bellance et al., 2013; Wargon et al., 2015). Our results show that PRA may increase tumorigenesis in both breast and prostate cancer by increasing AR-mediated transactivation of target genes containing the selective ARE and ERE. On the other hand, PRA may decrease oncogenic effects of the AR on target genes containing the classical ARE. Further studies are needed to investigate the physiological implications of these results.

To gain further insights into the results observed in Chapter 3, future studies could aim to understand the ligand-specific effects observed with $P_4$ and MPA in the different cell lines. Molecular docking simulations have previously been employed to examine conformational changes in the AR when bound to different ligands (Africander et al., 2014). Similar studies can thus be used to further investigate the conformational change induced in the PR isoforms with different ligands. In addition, it could be investigated whether the cell-specific effects observed in this study may be due to differential metabolism of the ligands and/or due to differential interaction(s) of transcriptional co-regulators with the steroid receptors in the two cell lines. Metabolism could be investigated using liquid chromatography tandem mass spectrometry. To investigate the interaction between specific co-regulators and the AR and/or PR isoforms, the mammalian two-hybrid assay, commonly used to investigate protein-protein interactions, could be employed (He and Li, 2008). This technique could also be employed to investigate the direct interaction that we hypothesise occurs between the AR and PRB or PRA. Förster resonance energy transfer (FRET) studies can also be used to confirm the results from the mammalian two-hybrid assay, by tagging the AR and PRB or PRA with the FRET coupled yellow fluorescent protein (YFP) and cyano fluorescent protein (CFP) (Sekar and Periasamy, 2003).

It would be interesting to determine whether the effects observed on the synthetic promoters in this study translate to a more physiologically relevant system. Using quantitative real-time polymerase chain reaction (qPCR), effects could be investigated on endogenous genes implicated in breast and/or prostate cancer development and progression. Examples of genes are the PR target gene, *GATA3* that

contains a PRE/classical ARE sequence within its promoters (Lai et al., 2009; Yin et al., 2012), the AR target gene, *PSA* that contains both a PRE/classical ARE sequence and a selective ARE sequence (Lai et al., 2009), and genes containing an ERE sequence such as the anti-apoptotic *bcl-2* and tumour-suppressive *p53* genes (Ikeda et al., 2015). Furthermore, to gain more insight into our hypothesis that both the AR and PR isoforms are responsible for the increased transactivation observed in Chapter 3, chromatin immunoprecipitation (ChIP) assays could be performed to firstly determine whether the AR or PRB or PRA are recruited to the various response elements in the promoters of the above-mentioned endogenous genes. The ChIP assay uses receptor-specific antibodies and primers recognizing specific DNA sequence to study the interaction between a protein and a specific DNA sequence *in vivo* (Gade and Kalvakolanu, 2012). To establish whether the AR and PRB or PRA are co-recruited to the promoter of these genes, sequential ChIP (re-ChIP) assays could be performed This technique is based on the same principle as the ChIP assay, but involves two immnoprecipitations with two different antibodies (Truax and Greer, 2012).

Finally, as we were unable to show the effects of the AR on breast and prostate cancer cell proliferation, and thus the effects of the PR isoforms on AR-mediated proliferation, future studies could aim to optimise the cell systems in terms of the amount of receptor to be transfected and experimental conditions such as incubation time. In addition, cell proliferation studies could be performed in cell lines expressing endogenous AR and PR, such as the T47D breast cancer cell line or the LNCaP prostate cancer cell line (Lai et al., 2009). The roles of the individual receptors could be determined by silencing the expression of the AR or PR using small interfering RNA (siRNA) technology.

In summary, the results from this study show that the PR can mimic AR transactivation function and that AR-PR crosstalk is indeed possible, occurring in a cell-, ligand- and PR concentration-specific manner. These results emphasise the fact that ligands may induce a conformational change within steroid receptors, which may lead to not only ligand-specific, but also promoter-specific effects, depending on the cellular milieu. Finally, although the results from this study are preliminary, the complexity of the role of steroid receptors in breast and prostate cancer are highlighted, and may fortify studies showing that the AR and PR mediate tumour progression and treatment failure in breast and prostate cancer.

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# Addendum A

# List of buffers and solutions

**2x Laemmli SDS-sample buffer**

100 mM Tris-HCl at pH 6.8

20% v/v glycerol

5% w/v SDS

0.1% w/v bromophenol blue

2% v/v β-mercaptoethanol

Adjust to a final volume of 25 ml using reverse osmosis (RO) water.

**Charcoal stripping buffer**

0.2 M sucrose

10 mM Hepes

1.5 mM $MgCl_2$ hexahydrate

0.25% w/v Norit-A charcoal

0.025% w/v Dextran

Adjust to a final volume of 1 L using autoclaved deionised water.

**Luria Bertani (LB) broth**

1% w/v NaCl

1% w/v tryptone

0.5% w/v yeast extract

Adjust to pH 7.5.

Adjust to a final volume of 1 L using RO water.

Sterilise by autoclaving.

**Passive lysis buffer**

0.2% v/v Triton X-100

10% v/v glycerol

2.8% v/v Tris-phosphate-EDTA

1.44 mM EDTA

Adjust to a final volume of 1 L using RO water.

**SDS-PAGE running buffer**

35 mM SDS

250 mM Tris-HCl

1.92 M glycine

Adjust to a final volume of 1 L using RO water.

**Transfer buffer**

25 mM Tris-HCl

192 mM glycine

10% v/v methanol

Adjust to a final volume of 1 L using RO water.

**Super optimal broth medium with catabolite repression (SOC)**

2% w/v tryptone

0.5% w/v yeast extract

10 mM NaCl

2.5 mM KCl

20 mM $MgCl_2$

20 mM glucose

Adjust to a final volume of 1 L using RO water and sterilise by autoclaving.

**Tris-EDTA (TE) buffer**

10 mM Tris/HCl

1 mM EDTA

Adjust to pH 8.0.

Adjust to a final volume of 100 ml using RO water.

# Addendum B

# Supplementary figures

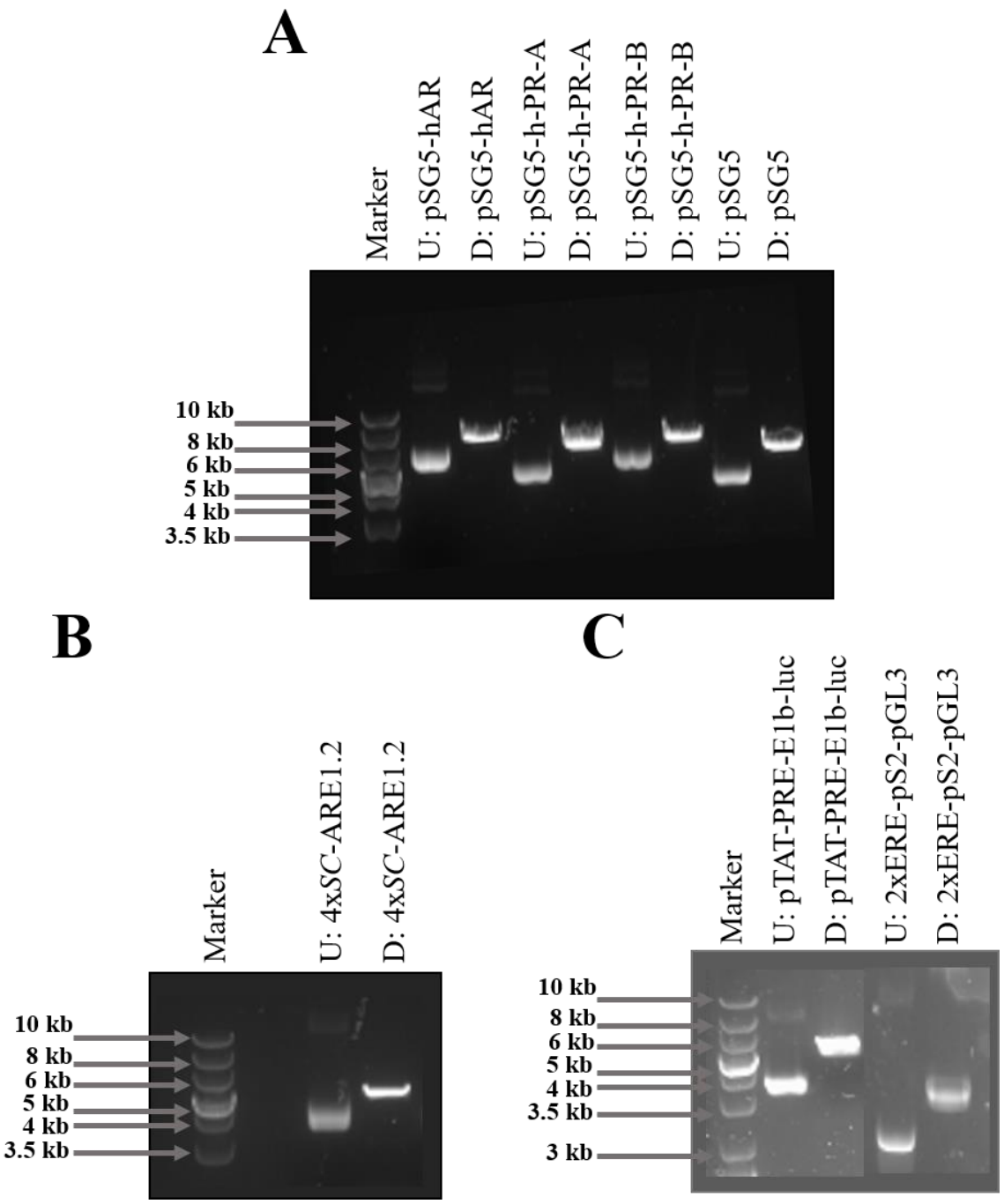


**Figure S1: Restriction enzyme digests of plasmid DNA confirm plasmid DNA integrity and size.** Digested and undigested plasmid DNA was separated on a 1% agarose gel electrophoresis. Lane 1 of A, B and C contains the O'GeneRuler 1kb DNA ladder (ThermoFisher Scientific, South Africa). A detailed description of each lane is provided above each figure, where 'U' represents the undigested plasmid and 'D' the digested plasmid.

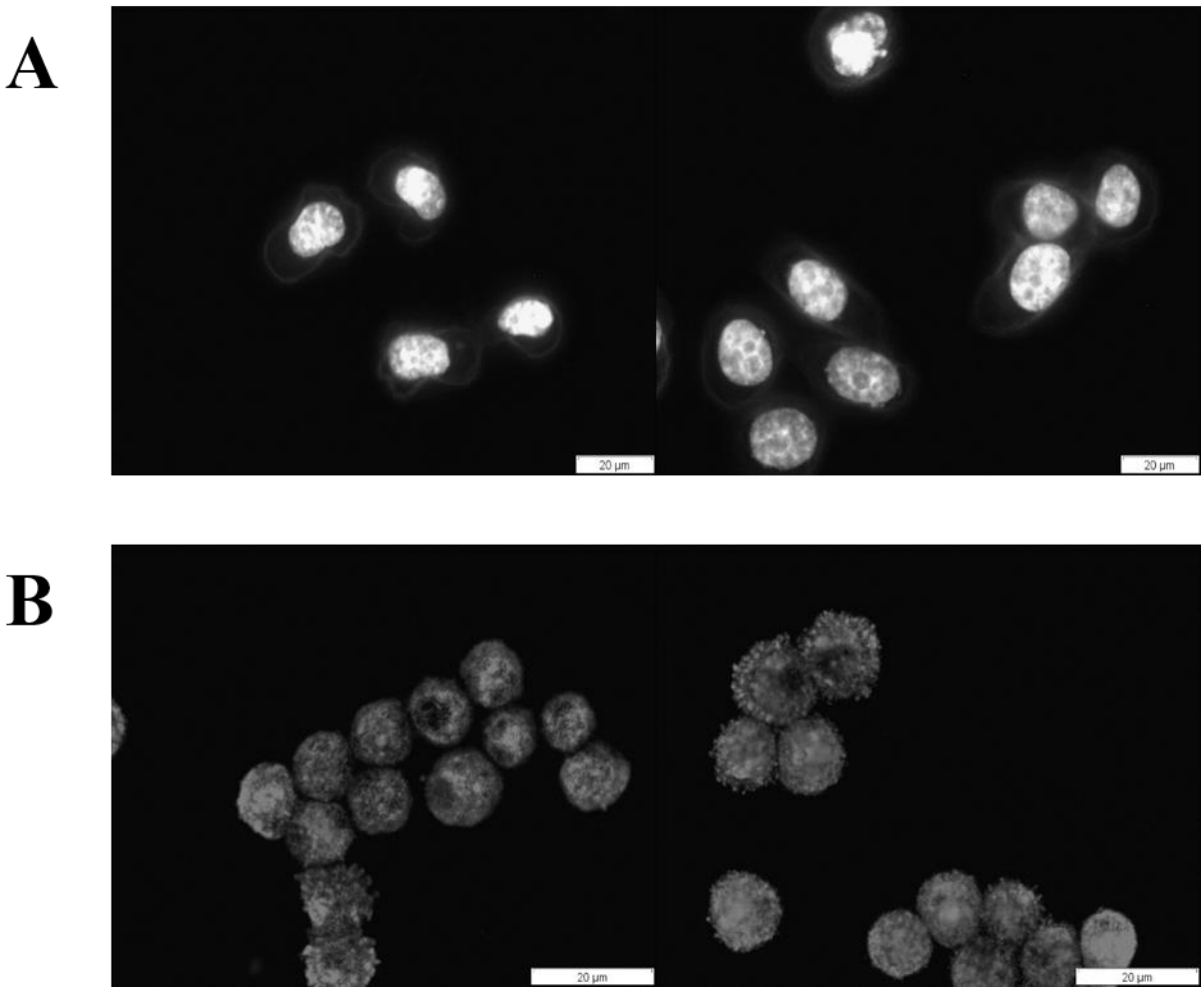


**Figure S2: The PC3 and MDA-MB-231 cell lines used for experiments are mycoplasma negative.** Representative images indicate that the PC3 (A) and MDA-MB-231 (B) cell lines are mycoplasma negative as determined by the Hoechst staining technique (Freshney, 2010). Mycoplasma negative cells were visualised using the Olympus IX81 inverted fluorescent microscope for live cell imaging at Stellenbosch University CAF.